Interactions Between Hepatitis C Virus and Proprotein Convertase Subtilisin/Kexin Type 9

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In Partial Fulfillment of the Requirements for the Degree of
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In the School of Public Health
University of Saskatchewan
Saskatoon, Saskatchewan, Canada

By
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ABSTRACT

Hepatitis C virus (HCV) is a small enveloped positive-sense single-stranded RNA virus that infects 2-3% of the world population. The majority of infected people develop chronic hepatitis, which results in severe liver damages. No HCV vaccine has been developed and the current antiviral regimens have some limitations such as high costs and not being effective in some difficult-to-treat patients.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease primarily produced in the liver. Its gene expression can be regulated by several transcription factors, such as sterol-regulatory element binding proteins (SREBPs), hepatocyte nuclear factor (HNF)-1, forkhead box O3 (FoxO3) and specificity protein 1 (Sp1). PCSK9 plays an important role in lipid homeostasis through facilitating the degradation of the low-density lipoprotein receptor (LDLR). It also exerts an antiviral effect on HCV. It can suppress HCV entry by reducing HCV receptors LDLR and cluster of differentiation 81. Although PCSK9 has been shown to inhibit HCV replication, the underlying mechanism has not been thoroughly characterized. Besides, the effects of PCSK9 on HCV translation and virion assembly/secretion have not been studied.

Since PCSK9 can regulate lipid levels and the HCV life cycle is closely connected with lipid metabolism, I hypothesized that PCSK9 has an inhibitory effect on the HCV life cycle. I first showed that PCSK9 does not affect HCV translation or virion assembly/secretion. However, an inhibitory effect of PCSK9 on HCV replication is shown by overexpressing or knocking down PCSK9 in HCV replicon cells. Then I demonstrated that PCSK9-induced LDLR degradation is not involved in HCV replication regulation using gain-of-function (D374Y) or loss-of-function (Δaa. 31-52) PCSK9 mutants for LDLR degradation. Moreover, the auto-cleavage of PCSK9 affects HCV replication since only uncleaved proPCSK9 suppresses HCV replication and cleaved PCSK9 does not have effect on HCV replication. Next, I found that PCSK9 can interact with several HCV proteins including NS5A. The PCSK9 interacting region of NS5A is aa. 95-215 in domain I. The interaction between PCSK9 and NS5A inhibits NS5A dimerization and HCV RNA binding to NS5A. Considering that NS5A dimerization and RNA binding activity of NS5A are required for HCV replication, the interaction between PCSK9 and NS5A could be a mechanism of the inhibitory effect of PCSK9 on HCV replication.

Since interferon (IFN) produced by innate immune system is important to clear viral infection and PCSK9 can inhibit HCV infection, I further hypothesized that PCSK9 affects HCV infection through regulating IFN production. I showed that PCSK9 suppresses IFNβ expression at the transcription and protein levels. The inhibitory effect of PCSK9 on IFNβ promoter/enhancer activity is mediated by positive regulatory domain IV in the IFNβ enhancer region where the activating transcription factor-2 (ATF-2)/c-Jun complex can bind. I demonstrated an interaction between PCSK9 and ATF-2. This interaction reduces ATF-2/c-Jun dimerization and ATF-2/c-Jun binding to IFNβ enhancer, which could explain how PCSK9 inhibits IFNβ expression. This is a novel function of PCSK9.

HCV can differently modulate transcription factors involved in PCSK9 expression including SREBPs, HNF-1 and FoxO3, but how HCV regulates PCSK9 expression remains unknown. In this study, I demonstrated that HCV can up-regulate PCSK9 promoter activity in the context of HCV infection and in HCV replicon cells. Among HCV viral proteins, NS2, NS3, NS3-4A, NS5A and NS5B enhance, and p7 or NS4B decreases PCSK9 promoter activity. I also showed that transcription factors SREBP-1c, HNF-1α and Sp1 increase PCSK9 promoter activity in HCV replicon cells, whereas SREBP-1a, HNF-1β and FoxO3 have an inhibitory effect.

In conclusion, I showed complex interactions between HCV and PCSK9. On one hand, HCV up-regulates PCSK9 promoter activity. On the other hand, PCSK9 inhibits HCV replication via the interaction with NS5A. It also suppresses IFNβ expression via the interaction with ATF-2, which may regulate HCV infection. This research advances the understanding of the regulation of PCSK9 and the effects of PCSK9 on viral infection and IFN expression, and may help to optimize anti-HCV treatments.

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DEDICATION

This work is dedicated to my parents

who supported and encouraged me during my studies.

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LIST OF ABBREVIATIONS

AD	Alzheimer’s disease
AH	amphipathic helix
Ahx	aminohexanoic acid
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP-1	activating protein-1
Apo	apolipoprotein
ApoER2	apolipoprotein E receptor 2
ATF-2	activating transcription factor-2
Aza	5-Aza-2'-deoxycytidine
BACE1	β-site amyloid precursor protein-cleaving enzyme 1
βTrCP	β-transducing repeat-containing protein
bZIP	basic region-leucine zipper
CA	catalytic domain
CBP	cAMP-response element-binding protein-binding protein
CD	cluster of differentiation
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CHRD	cysteine- and histidine-rich domain
CK2	casein kinase 2
CKI	cyclin-dependent kinase inhibitor
co-IP	co-immunoprecipitation

DAA	direct-acting antiviral
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DGAT1	diacylglycerol acyltransferase 1
DMEM	Dulbecco's Modified Eagle Medium
DMV	double membrane vesicle
DN	dominant negative
E6AP	E6-associated protein
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
eIF	eukaryotic initiation factor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ENaC	epithelial Na^+ channel
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAS	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FoxO3	forkhead box O3
FXR	farnesoid X receptor
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase

GUSB	β-glucuronidase
HAT	histone acetyltransferase
HBV	hepatitis B virus
HC	heavy chain
HCV	hepatitis C virus
HCVcc	cell culture-derived HCV
HCVpp	HCV pseudoparticle
HDAC	histone deacetylase
HINFP	histone nuclear factor P
HIV	human immunodeficiency virus
HNF	hepatocyte nuclear factor
hPLIC1	human homolog 1 of protein linking integrin-associated protein and cytoskeleton
HR	hinge region
HSPG	heparin sulfate proteoglycan
hVAP-33	human vesicle-associated membrane-associated protein of 33 kDa
IFN	interferon
IFNL4	interferon λ-4
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	interleukin
IP-10	interferon γ-inducible protein-10
IRES	internal ribosomal entry site
IRF	interferon regulatory factor

ISG	interferon-stimulated gene
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
LC	light chain
LCS	low-complexity sequence
LD	lipid droplet
LDL	low-density lipoprotein
LDLC	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
L-SIGN	liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
luLD	luminal lipid droplet
LVP	lipoviroparticle
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MMV	multiple membrane vesicle
MOI	multiplicity of infection
MTTP	microsomal triglyceride transfer protein
MyD88	myeloid differentiation primary response 88
NANBH	non-A, non-B transfusion-associated hepatitis
NES	nuclear export signal
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization signal
NOS2A	nitric oxide synthase 2A

NPAT	nuclear protein of the ataxia telangiectasia mutated locus
NPC1L1	Niemann-Pick C1-like 1
NRD	negative regulatory domain
NS	not significant
OAS	oligoadenylate synthetase
ORF	open reading frame
PACE4	paired basic amino acid cleaving enzyme 4
PAMP	pathogen-associated molecular pattern
PC	proprotein convertase
PCSK9	proprotein convertase subtilisin/kexin type 9
PD	prodomain
PEG	polyethylene glycol
PEG-IFNα	pegylated interferon α
PKA	protein kinase A
PKC	protein kinase C
PKR	protein kinase R
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
pRb	retinoblastoma tumor suppressor protein
PRD	positive regulatory domain
PRK2	protein kinase C-related kinase 2
PRMT	protein arginine methyltransferase
PRR	pathogen recognition receptor
PTEN	phosphatase and tensin homolog deleted on chromosome 10

PTM	post-translational modification
qPCR	quantitative real-time PCR
RdRp	RNA-dependent RNA polymerase
RFP	red fluorescent protein
RG	arginine-glycine
RIG-I	retinoic acid-inducible gene I
RIP	RNA and protein immunoprecipitation
ROS	reactive oxygen species
RT	reverse transcription
RT-qPCR	reverse transcription quantitative real-time PCR
S1P	site 1 protease
SGR	subgenomic replicon
SKI	subtilisin/kexin isozyme
SLCA	split luciferase complementation assay
SNP	single-nucleotide polymorphism
SP	signal peptide
Sp1	specificity protein 1
SR-B1	scavenger receptor class B type 1
SRE	sterol-regulatory element
SREBP	sterol-regulatory element binding protein
SUMO	small ubiquitin-related modifier
SVR	sustained virological response
TC-PTP	T-cell protein tyrosine phosphatase
TfR	transferrin receptor

TG	triglyceride
TLR	toll-like receptor
TM	transmembrane domain
TNF	tumor necrosis factor
TRAF2	TNF receptor-associated factor 2
TRIF	toll/IL-1 receptor homology domain-containing adaptor inducing interferon β
TRRAP	transformation/transactivation domain-associated protein
UTR	untranslated region
VLDL	very-low-density lipoprotein

1.0 LITERATURE REVIEW

1.1 Hepatitis C Virus

1.1.1 HCV identification

Hepatitis C virus (HCV) is a blood-borne infectious virus, which was first identified in 1989 and which is responsible for most non-A, non-B transfusion-associated hepatitis (NANBH) cases (Choo et al., 1989; Feinstone et al., 1975). For a long time, the NANBH agent could not been identified using traditional immunological or serological methods. Choo et al. constructed a cDNA library in bacteriophage λgt11 using DNA and RNA isolated from NANBH-infected chimpanzee plasma, and expressed these constructs in *E. coli*. After using chronic NANBH patient serum to screen, a positive cDNA clone was identified. It was derived from exogenous positive-stranded RNA and contained more than 10,000 nucleotides. Besides, the protein encoded by this cDNA clone specifically interacted with NANBH patient serum, which confirmed the isolation of the NANBH agent. This identified NANBH agent was what we now know as HCV (Choo et al., 1989; Choo et al., 1990; Houghton, 2009). Due to the development of PCR technology, sequence comparisons of HCV isolates classify HCV into the genus *Hepacivirus* in the family *Flaviviridae*. There are seven distinct but related genotypes, with hundreds of subtypes (Murphy et al., 2015; Robertson et al., 1998; Simmonds, 1995). The nucleotide and amino acid differences among genotypes and subtypes are about 30% and 20%, respectively (Scheel et al., 2012).

1.1.2 HCV epidemiology

Nowadays, about 2-3% of the world population (130-170 million) are estimated to be infected with HCV and there are about 4 million new infection cases each year (Mohamed et al., 2015; Torresi et al., 2011; Westbrook and Dusheiko, 2014). HCV mainly transmits through percutaneous exposures to blood and the transmission routes include transfusion of blood and blood products, organ transplant, intravenous drug use and reuse of invasive medical instruments. Sexual transmission and mother-to-child transmission are less common. Occupational exposure is also a risk factor for health care workers (Alter, 2007; Morozov and Lagaye, 2018; Ponde, 2011). HCV/human immunodeficiency virus (HIV) or HCV/hepatitis B virus (HBV) co-infection is common due to the same transmission routes of these viruses (Fernandez-Montero and Soriano,

2012). In developed countries, the prevalence is less than 2%. Higher prevalence is reported in regions including Central Asia, East Asia, South Asia, West and Central Sub-Saharan Africa, North Africa and the Middle East (Petruzziello et al., 2016). Egypt has the highest prevalence (more than 10%) due to the iatrogenic transmission in parenteral anti-schistosomiasis treatment from 1950 to 1980 (El Kassas et al., 2018). As the most prevalent genotypes, HCV genotype 1 accounts for almost half (49.1%) of HCV-infected population and is predominant in North America, Caribbean, Latin America, Asia (except for South Asia), Australasia and Europe. Genotype 2 (11.0% of infection cases) mainly occurs in East Asia, West and East Sub-Saharan Africa. Genotype 3 (17.9% of infection cases) widely distributes in South Asia, Tropical Latin America, Australasia and Europe. Genotype 4 (16.8% of infection cases) is predominant in Central Sub-Saharan Africa, North Africa and the Middle East. Genotype 5 and 6 account for less than 5% of infection cases and mainly occur in Southern Sub-Saharan Africa and Southeast Asia, respectively (Petruzziello et al., 2016). Genotype 7 has origin in the Democratic Republic of Congo and only a few patients have been identified with this genotype (Murphy et al., 2015).

1.1.3 HCV infection clinical outcome

HCV infection is often asymptomatic in the initial six months (acute phase). About 15-30% of acute HCV infection cases show mild and unspecific symptoms, such as nausea, fatigue, jaundice, abdominal pain and loss of appetite. Diagnosis made during this period is characterized by the appearance of HCV RNA in the blood as early as within two weeks of infection, followed by elevated alanine aminotransferase (ALT) level and anti-HCV antibody seroconversion within 20-150 days of infection (Boesecke et al., 2012; Hajarizadeh et al., 2013; Irving et al., 2008). Since the majority of acute infected people are asymptomatic, diagnosis in early-stage HCV infection is often neglected and therefore treatment is delayed. Only 20-30% of infected people can spontaneously control and clear the virus, which means HCV RNA cannot be detected. It usually happens within the first three to six months of infection. Many host factors are associated with spontaneous clearance rate, including age, sex, symptom, genetic factors (interleukin (IL)-28B gene and interferon λ-4 (IFNL4) gene single-nucleotide polymorphisms (SNPs)), interferon γ-inducible protein-10 (IP-10) serum level, the presence of HCV specific neutralizing antibodies. HCV RNA level, HCV genotype and the genetic diversity of HCV quasispecies are the viral

factors that are also related to viral clearance (Chung, 2005; Grebely et al., 2011; Sharma and Feld, 2014).

The majority of HCV-infected people develop into chronic hepatitis C such that HCV RNA is continuously detected after six months of infection. At this phase, spontaneous clearance is rare. Chronic HCV infection can result in liver fibrosis, steatosis, cirrhosis and hepatocellular carcinoma, and may eventually lead to liver failure. This progress develops slowly and may take 30-40 years (Maasoumy and Wedemeyer, 2012; Zoulim et al., 2003). Fibrosis progression rate varies and can be accelerated by many factors, such as age, sex, elevated ALT level, chronic HIV or HBV co-infection, diabetes, insulin resistance, obesity and excessive alcohol consumption (Chen and Morgan, 2006; Hajarizadeh et al., 2013). Liver steatosis is prevalently correlated with HCV genotype 3 infection and contributes to fibrosis progression (Maasoumy and Wedemeyer, 2012). Cirrhosis progression is linked with age, excessive alcohol consumption and some genetic factors including seven SNPs. It is always protracted and clinically silent until the end-stage. The incidence doubles after 30 years of HCV infection (41%) compared to after 20 years (16%) (Chen and Morgan, 2006; Hajarizadeh et al., 2013). About 1-3% of chronic HCV infection develops into hepatocellular carcinoma, and old age, male sex, HBV co-infection, diabetes and excessive alcohol consumption may increase the risk. Chronic HCV infection with cirrhosis has a higher hepatocellular carcinoma incidence than non-cirrhotic HCV chronic infection (Hajarizadeh et al., 2013; Maasoumy and Wedemeyer, 2012; Yamashita et al., 2011). Some extrahepatic manifestations, such as lymphoproliferative disorders, atherosclerosis, vascular diseases, renal insufficiency, diabetes, insulin resistance and neurological dysfunctions, are also associated with chronic HCV infection (Cacoub et al., 2014; Zampino et al., 2013).

1.1.4 HCV genome

HCV is an enveloped positive-sense single-stranded RNA virus that is 55-65 nm in size. Its 9.6 kb genome contains a large open reading frame (ORF) flanked by a 5' untranslated region (UTR) and a 3'UTR (Figure 1.1). The ORF encodes a polyprotein precursor of about 3,000 amino acids. Both the 5'UTR and 3'UTR are highly conserved (Chevaliez and Pawlotsky, 2006; Sharma, 2010). The 5'UTR consists of four domains with 341 nucleotides. Domain I and II are vital for HCV replication. An internal ribosomal entry site (IRES) spanning from domain II to IV plus a few nucleotides from coding region of core protein plays an important role in cap-independent

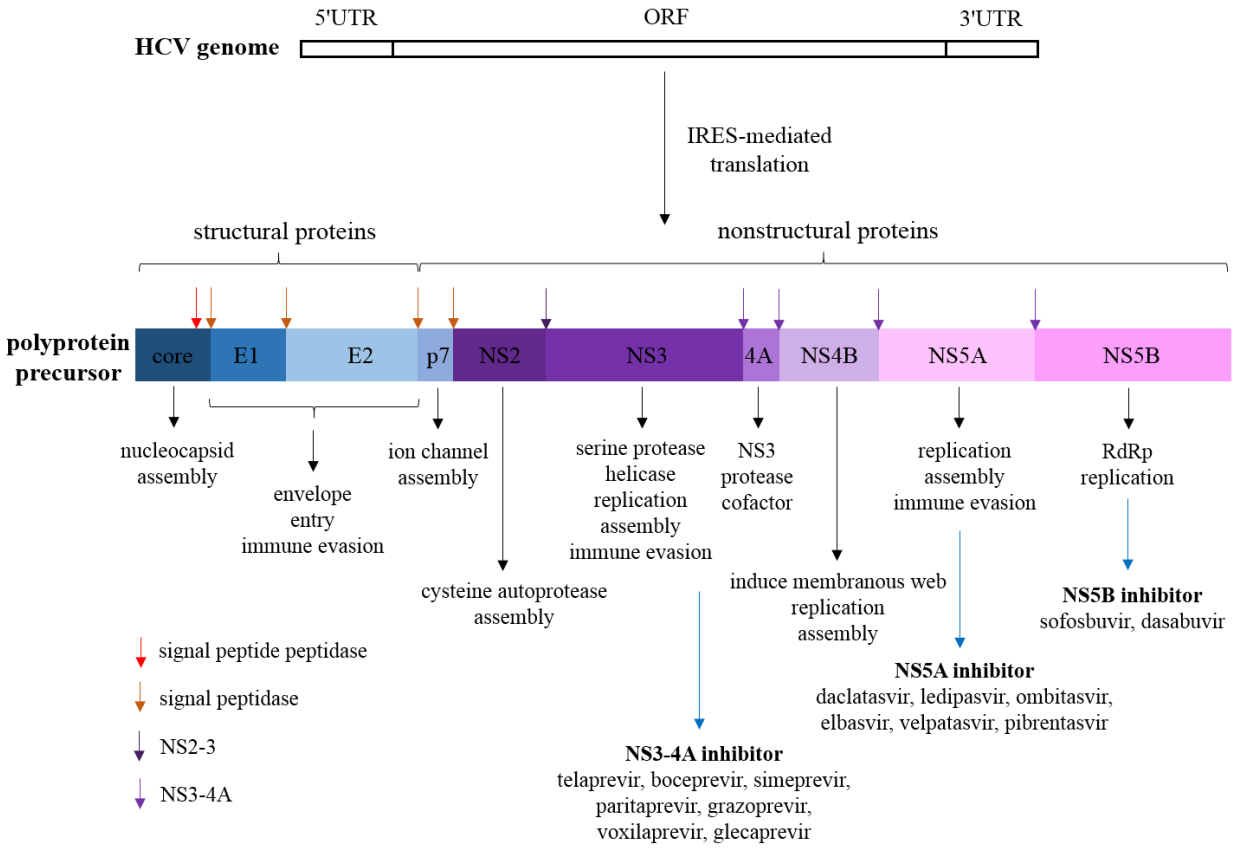

Figure 1.1 HCV genome and HCV polyprotein processing. The HCV genome consists of a 5'UTR, an ORF and a 3'UTR. The polyprotein precursor produced by IRES-mediated translation is cleaved into 10 viral proteins by host and viral proteases. The functions of HCV proteins and FDA-approved DAAs are indicated.

translation (Lukavsky, 2009; Morozov and Lagaye, 2018). The 3'UTR functions in viral replication and translation. It is composed of a variable region (40 nucleotides), a poly(U/UC) tract (30-80 nucleotides according to HCV genotypes) and a highly conserved X-tail containing three stem loop structures (SL1, SL2 and SL3) (98 nucleotides) (Bradrick et al., 2006; Morozov and Lagaye, 2018).

1.1.5 HCV proteins

1.1.5.1 Permission to use

This section contains a modified version of our previously published review article: Hundt, J., Li, Z., and Liu, Q. (2013). Post-translational modifications of hepatitis C viral proteins and their biological significance. *World J Gastroenterol* 19, 8929-8939 (https://www.wjgnet.com/1007-9327/full/v19/i47/8929.htm). As per *World Journal of Gastroenterology* policy, no further permission is required for reuse or modification by the authors. Details are available at https://www.wjgnet.com/1007-9327/Nav/456.

1.1.5.2 Proteolytic cleavage of HCV polyprotein

Proteolytic processing of the HCV polyprotein encoded by the ORF giving rise to single viral proteins represents an initial step in viral protein modification. There are nine defined proteolytic cleavage sites within the HCV polyprotein precursor, resulting in the generation of at least ten non-overlapping proteins, including structural proteins core, E1 and E2, and nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Figure 1.1). Additional viral protein products might be produced by alternative ORFs discovered within the HCV genome (Branch et al., 2005; Chevaliez and Pawlotsky, 2006; Moradpour et al., 2007; Suzuki et al., 2007).

Proteolytic processing of the polyprotein precursor occurs co- and post-translationally involving cellular as well as viral proteases (Figure 1.1) (Penin et al., 2004). The structural proteins are cleaved off the polyprotein precursor by host cellular signal peptidase located in the endoplasmic reticulum (ER) of the host cell, while the nonstructural proteins are released from the polyprotein precursor by viral proteases NS2-3 and NS3-4A (Suzuki et al., 2007). The core protein is found to be additionally cleaved inside the ER membrane by host cellular signal peptide peptidase, thus yielding the mature core variant (McLauchlan et al., 2002). This step

leads to the release of core from the ER and its trafficking to lipid droplets (LDs) which are believed to serve as a platform for HCV particle assembly (Boson et al., 2011). E2, p7 and NS2 are first generated as an E2-p7-NS2 precursor protein. The E2-p7-NS2 precursor is proteolytically processed at the p7-NS2 junction efficiently whereas the E2-p7 junction gets cleaved less frequently, hence resulting in the presence of E2 and p7 proteins as well as the non-cleaved E2-p7 variant in infected cells (Dubuisson, 2000). The NS2-3 autoprotease cleaves at the NS2-NS3 junction and the NS3-4A protease cleaves at the sites between NS3 and NS4A, NS4A and NS4B, NS4B and NS5A, and NS5A and NS5B. Proteolytic processing by the NS3-4A complex follows a certain order that the cleavage first happens *in cis* at the NS3-NS4A junction, then rapidly *in trans* at NS5A-NS5B followed by proteolysis at NS4A-NS4B, and finally at NS4B-NS5A. NS3 to NS5B mainly function in HCV genome replication (Chevaliez and Pawlotsky, 2006; Morikawa et al., 2011). Proteolytic processing releases structural and nonstructural HCV viral proteins that take part in different stages of the HCV life cycle.

1.1.5.3 The nucleocapsid core protein

HCV core protein is the most conserved viral protein among different HCV genotypes. It constitutes the viral nucleocapsid that encapsidates the viral RNA genome, and is essential for virus particle assembly (Dubuisson, 2007; Khaliq et al., 2011b). HCV core also exerts several regulatory functions, such as cellular transcription, virus-induced transformation and signal transduction, and can predispose to steatosis and hepatocellular carcinoma. Moreover, the core is significantly involved in virus-mediated pathogenesis. It is able to modulate apoptosis and cell growth, but also up-regulates reactive oxygen species (ROS) production and has a possible immunoregulatory role (Jahan et al., 2012; Khaliq et al., 2011b).

The complete core protein is composed of three domains: an N-terminal hydrophilic domain that is essential for RNA binding and homo-oligomerization, a C-terminal hydrophobic domain that associates with LDs and is involved in proper folding, and a hydrophobic signal sequence tail that can target E1 to the ER membrane (Boulant et al., 2006; Khaliq et al., 2011b; Murray et al., 2007; Roingeard and Hourioux, 2008). Unlike other HCV proteins, core protein liberation from HCV polyprotein precursor needs sequential proteolytic processing. Following cleavage from HCV polyprotein at the core-E1 junction by host cellular signal peptidase, the immature core protein (23 kDa) is additionally cut by signal peptide peptidase within its hydrophobic C-

terminus to release mature N-terminal amino acids 173-179 core protein (21 kDa) and dissociate core from the ER membrane (Chevaliez and Pawlotsky, 2006; Khaliq et al., 2011b; Kopp et al., 2010). The exact C-terminus of the mature core has not been identified yet in mammalian cells, even though it is reported to be Phe^{177} or Leu^{179} in insect cells (Okamoto et al., 2008). This further processing by signal peptide peptidase relies on previously correct cleavage by signal peptidase and the sequential processing controls viral protein production rate (Pene et al., 2009). Only the mature form of core can attach to LDs and interact with NS5A that transports HCV genome RNA to core (McLauchlan, 2000, 2009). Therefore, core maturation by signal peptide peptidase cleavage plays a critical role in virus assembly and regulation of the HCV life cycle.

It has been demonstrated that disulfide bonds in nucleocapsid proteins of viruses with icosahedral structure play a role in virus assembly and capsid structure stabilization (Kushima et al., 2010). Since HCV is packaged into a similar spherical structure, its nucleocapsid might resemble the same organization (Ishida et al., 2001). Mutation analysis discovered that mature core forms a dimeric membrane protein which is linked by disulfide bond at Cys^{128}. This disulfide bond formation stabilizes capsid structure and strengthens the interaction between core and membranes, and is critical for virus assembly and virion production, However, the disulfide bond in core has no effect on HCV RNA replication, the association with LDs or other functions of core (Kushima et al., 2010). Because of the low mutation rate of Cys^{128}, drugs targeting Cys^{128} disulfide bond formation may be considered as a candidate to inhibit HCV virion formation.

Phosphorylation is a common type of post-translational modifications (PTMs), which is also observed in HCV core protein. The phosphorylation of the core by protein kinase A (PKA) at Ser^{53} and Ser^{116} and by protein kinase C (PKC) at Ser^{53} and Ser^{99} was reported both *in vitro* using purified proteins and in core-transfected Huh-7 and HepG2 cells. Ser^{99} and Ser^{116} are the major and predominant sites with low phosphorylation efficiency (Lu and Ou, 2002; Shih et al., 1995). The phosphorylation at these two sites is critical for inhibition of HBV gene expression and replication in Huh-7 cells, but the detailed *trans*-suppression mechanism of HCV core remains unclear. The same study showed that only truncated core is phosphorylated by PKC, suggesting structural conformation might be a prerequisite for phosphorylation (Shih et al., 1995). The phosphorylation at Ser^{116} by PKA is responsible for the repressive activity of the core on cyclin-dependent kinase inhibitor (CKI) p21 promoter (Jung et al., 2001). Reduced p21 may interfere with p53-driven repair mechanism in cell cycle, which may facilitate tumorigenesis. Another role

of phosphorylation of the core might be involved in modulating nuclear localization of core, although controversial results have been reported (Lu and Ou, 2002; Shih et al., 1995). The nuclear core is involved in regulating host gene transcription (Gomez-Gonzalo et al., 2004).

Moreover, HCV core also undergoes several other types of PTMs. For example, the ubiquitination of core protein by E3 ubiquitin ligase E6-associated protein (E6AP) preferentially at N-terminal lysine residues induces the degradation of core in the cytoplasm by the ubiquitin-proteasome pathway, which could control HCV virion production and have an antiviral effect. The interaction region between core and E6AP is located between amino acids 58-71 of the core protein, which are highly conserved in all HCV genotypes (Moriishi et al., 2010; Shirakura et al., 2007; Suzuki et al., 2009). Palmitoylation of the core at Cys^{172} plays a vital role in targeting the core to smooth ER and ER-associated LDs, but does not affect signal peptide peptidase proteolytic cleavage-induced maturation and LD accumulation. Importantly, it also affects HCV assembly and production (Majeau et al., 2009).

1.1.5.4 The envelope glycoproteins E1 and E2

HCV envelope glycoproteins E1 and E2 play important roles in virus entry and immune evasion (Zeisel et al., 2011). In infected cells, E1 and E2 are either found as noncovalent heterodimers which are mainly localized to the ER, or as disulfide-linked aggregates, which were originally thought to represent misfolded protein complexes (Drummer et al., 2003; Dubuisson and Rice, 1996; Op De Beeck et al., 2004; Vieyres et al., 2010). Heterodimers and oligomers of E1 and E2 are also found in infectious virus particles, whose structure is stabilized by disulfide bonds (Fraser et al., 2011; Helle et al., 2010; Vieyres et al., 2010).

Both E1 (33-35 kDa) and E2 (70-72 kDa) proteins consist of a large N-terminal ectodomain and a C-terminal hydrophobic transmembrane anchor (Chevaliez and Pawlotsky, 2006). PTMs of HCV envelope proteins include the attachment of glycans and the formation of disulfide bridges (Goffard et al., 2005; Lavie et al., 2007). Glycans attached to HCV envelope proteins are shown to modulate virus entry by modifying their receptor binding affinity or fusion activities. They are also involved in protein folding and play a key role in immune evasion by masking potential antigenic sites from binding of neutralizing antibodies (Goffard et al., 2005; Helle et al., 2007; Meunier et al., 1999). Because glycosylation sites within HCV glycoproteins are rather highly

conserved, glycosylation mutants are considered as immunogens to induce a potent antibody response against HCV (Falkowska et al., 2007).

There are four to five N-glycosylation sites in E1 and up to 11 N-glycosylation sites in E2 (Goffard et al., 2005; Goffard and Dubuisson, 2003; Helle et al., 2007; Slater-Handshy et al., 2004). N-linked glycosylation occurs at asparagine residues and the consensus sequence is Asn-X-Ser/Thr (Goffard et al., 2005; Kornfeld and Kornfeld, 1985). Mass spectrometric analysis of E2 revealed that this protein is mainly modified by high-mannose type oligosaccharides and more complex glycan types are observed for just two glycosylation sites within E2 (Iacob et al., 2008). E1 is believed to be modified only by high-mannose type oligosaccharides since a restricted localization of E1-E2 heterodimers to the ER is confirmed by immunofluorescence (Goffard and Dubuisson, 2003). However, more complex type glycosylations generally occur in the *cis*-Golgi compartment, where a small population of E2 protein has been detected by immunofluorescence (Martire et al., 2001). On the other hand, the attachment of complex glycans can happen during the release of viral particles via the exocytotic pathway, which involves the Golgi apparatus. Interestingly, due to the differences in the assembly process, more mature glycoproteins containing complex type glycans could have been observed with HCV pseudoparticles (HCVpp) compared to cell culture-derived HCV (HCVcc) particles (Vieyres et al., 2010). HCVpp is found to be assembled in post-Golgi compartments (Sandrin et al., 2005), while HCVcc assembly takes place in ER-derived compartments (Miyanari et al., 2007). HCVpp glycoproteins might also be more accessible to Golgi glycosyltransferases than HCVcc glycoproteins, which are components of high-order virion complexes (Vieyres et al., 2010). Differences in the glycosylation pattern of HCVpp and HCVcc might be relevant for studying HCV immune evasion strategies.

Furthermore, the carbohydrate composition of envelope glycoproteins vary to some extent depending on the cell line the virus infected (Etchison and Holland, 1974). Changes in the glycosylation pattern of HCV glycoproteins have a major impact on virus particle assembly, entry and immunogenicity (Goffard et al., 2005; Slater-Handshy et al., 2004), thus affecting virus pathogenesis and virulence. Mutations of glycosylation sites N1 and N4 in HCV glycoprotein E1 (E1N1, E1N4) as well as N8 and N10 in HCV glycoprotein E2 (E2N8, E2N10) strongly interfere with the incorporation of both envelope proteins into HCVpp, suggesting the importance of these sites for protein folding and E1-E2 heterodimerization (Goffard et al., 2005; Helle et al., 2010). Additionally, mutation of glycosylation site E2N2 or E2N4 leads to the decreased infectivity of

HCVpp, confirming a role of both glycans in virus entry (Goffard et al., 2005). Moreover, glycans at positions E2N1, E2N6 and E2N11 are shown to decrease the binding affinity of E2 to the cluster of differentiation (CD) 81 receptor and to reduce the sensitivity of pseudotyped HCV particles to antibody neutralization, hence contributing to humoral immune evasion (Helle et al., 2007). These findings are supported and extended by studies with HCVcc glycosylation mutants (Helle et al., 2010). Apparently, glycosylation sites E2N1, E2N2, E2N4 and E2N6 seem to surround the CD81 receptor binding site within E2, therefore "protecting" this site from recognition by neutralizing antibodies. Helle et al. provided structural evidence for glycans attached to HCV envelope proteins to modulate the humoral immune response (Helle et al., 2010).

Besides N-glycosylation, little information is available on O-glycosylation of HCV E1 or E2. Supposedly, there is one potential O-glycosylation site within E1 and four potential O-glycosylation sites within E2 (Falkowska et al., 2007). So far, three O-linked glycosylation sites in E2 have been shown to be important for HCV entry, with two of them apparently decreasing E2 binding affinity to CD81 receptor (Falkowska et al., 2007).

Virion-associated HCV glycoproteins are assembled into large oligomeric protein complexes which are stabilized by disulfide bonds (Helle et al., 2010; Vieyres et al., 2010). These complexes are able to bind conformation-sensitive neutralizing antibodies and recombinant CD81 (Vieyres et al., 2010), and therefore can be considered functionally significant rather than the result of a misfolding event.

Proper folding of glycoprotein E1 is dependent on E2 co-expression and *vice versa* (Brazzoli et al., 2005; Michalak et al., 1997). E1 and E2 consist of 8 and 18 highly conserved cysteine residues, respectively (Fraser et al., 2011). Structural information is mainly available for HCV E2 protein, where nine intramolecular disulfide bonds have been identified (Krey et al., 2010). Because of the difficulties in expressing E1 in the absence of E2, disulfide arrangement of cysteine residues in E1 has not been determined (Fraser et al., 2011). Beside their apparent impact on virus particle structure and infectivity, it is conceivable that disulfide-linked glycoprotein oligomers may play an active role in HCV budding by assisting protein-protein interactions (Vieyres et al., 2010). Furthermore, it is possible that the presence of disulfide bridges in HCV envelope proteins could be responsible for the lack of sensitivity of HCVcc to low pH treatment (Tscherne et al., 2006). This suggests their direct influence on virus internalization by affecting the presentation of HCV fusion peptide (Vieyres et al., 2010).

Additionally, the impact of disulfide rearrangement and the oxidation state of cysteine residues in E1 and E2 glycoproteins on HCV entry and membrane fusion was confirmed by Fraser et al. (Fraser et al., 2011). Here the presence of free thiol groups has been shown to be essential for HCV infectivity.

Altogether, PTMs of HCV glycoproteins by glycosylation and disulfide bond formation have a strong impact on several steps in viral life cycle, more specifically entry, fusion of viral membrane with the host cell's endosomal membrane and budding.

1.1.5.5 The ion channel complex protein p7

HCV p7 represents a 7 kDa small integral membrane protein, which is able to oligomerize and form proton channels within the HCV particle envelope. The precise role of p7 in the HCV life cycle has not been determined, even though it has been shown to be essential for infection, but not for viral replication (Lohmann et al., 1999; Sakai et al., 2003; Vieyres et al., 2013). Due to incomplete or delayed proteolytic processing, the generation of a p7 species linked to the E2 glycoprotein has been observed. The role of E2-p7 precursor during HCV infection is not known so far. However, it is speculated that E2-p7 might be involved in regulating the production of native p7 and formation of ion channel complexes (Carrere-Kremer et al., 2004). The optimal cleavage at the E2-p7 junction is shown to be important for virus production probably due to the increased NS2-associated virus assembly complex formation in close proximity of LDs. It also enhances NS2 interaction with NS3 and E2, but does not affect HCV genome replication (Shanmugam and Yi, 2013).

Structural analysis revealed that HCV p7 protein consists of two membrane-spanning α-helices connected by a short cytoplasmic loop (Khaliq et al., 2011a). PTMs of p7 have not been demonstrated.

1.1.5.6 The cysteine autoprotease NS2

HCV NS2 is a 23 kDa transmembrane protein (Yamaga and Ou, 2002). Together with the N-terminal domain of NS3, NS2 forms the NS2-3 autoprotease. The NS2-3 cysteine autoprotease is a zinc-dependent metalloprotease that cleaves the HCV polyprotein at the NS2-NS3 junction. After its self-cleavage from NS3, NS2 is quickly degraded (Schregel et al., 2009; Welbourn and Pause, 2007). Like p7, NS2 is known to be essential for virus assembly. Even though NS2 is part

of the HCV replication complex which is composed of NS2, NS3, NS4A, NS4B, NS5A and NS5B, NS2 is not essential for virus replication (Jirasko et al., 2008; Jones et al., 2007; Lohmann et al., 1999). The interaction of NS2 with E1, E2, NS3 and NS5A results in co-localization of these viral proteins to dot-like structures near LDs which are the sites for virus particle assembly (Ma et al., 2011; Shanmugam and Yi, 2013). Moreover, proper cleavage at the NS2-NS3 junction is important for an active HCV replication complex formation, but is not required for NS3 protease activity (Welbourn et al., 2005; Wright-Minogue et al., 2000). Other functions linked to NS2 include the inhibition of apoptosis and modulation of host cellular gene transcription (Dumoulin et al., 2003; Erdtmann et al., 2003; Oem et al., 2008a; Oem et al., 2008b).

The highly hydrophobic N-terminus of NS2 consists of three transmembrane segments which form the protein membrane binding domain (Jirasko et al., 2010). No attachment of fatty acids or prenyl groups by modifications typically involved in membrane targeting, like farnesylation, myristoylation, palmitoylation or prenylation (Resh, 2006), has been associated with membrane anchoring of NS2 so far. Though NS2 is located to the ER membrane, the protein is not glycosylated (Franck et al., 2005). The protease activity of NS2 is located within its C-terminal domain which is able to homodimerize and thus creates two composite active sites (Lorenz et al., 2006). Regarding the role of NS2 in HCV particle formation, the overall structural integrity rather than the protease activity of NS2 itself appears to be crucial (Dentzer et al., 2009; Phan et al., 2009).

The C-terminal globular domain of NS2 facing towards the cytoplasm of the infected cell is shown to be modified by phosphorylation. Phosphorylation of NS2 is presumably mediated by host cellular casein kinase 2 (CK2) (Franck et al., 2005). NS2 is a short-lived protein that is rapidly degraded by the proteasome. Proteasome-mediated degradation of NS2 is regulated in an ubiquitin-independent manner by phosphorylation within its C-terminal domain. Ser^{168}, as part of a CK2 consensus recognition site (Ser/Thr-X-X-Glu), is shown to be vital for NS2 degradation. It is highly conserved among all HCV genotypes and single point mutation of Ser^{168} confers resistance to NS2 degradation (Franck et al., 2005). Therefore, phosphorylation of NS2 is strongly connected to its abundance within the host cell and can have a strong impact on HCV pathogenesis, more particularly on assembly and virion production.

1.1.5.7 The serine protease and helicase NS3-4A complex

HCV NS3-4A is a noncovalent complex composed of the 69 kDa serine protease NS3 and its 6 kDa cofactor NS4A (Chevaliez and Pawlotsky, 2006; Moradpour and Penin, 2013). The NS3-4A mediated cleavage releases NS3, NS4A, NS4B, NS5A and NS5B from the HCV polyprotein in a specific order. The NS3-4A protease complex also has three identified cellular targets so far, including mitochondrial antiviral signaling protein (MAVS), T-cell protein tyrosine phosphatase (TC-PTP) and toll/IL-1 receptor homology domain-containing adaptor inducing interferon β (TRIF), which may be involved in the development of persistent infection and hepatocellular carcinoma (Moradpour et al., 2007; Morikawa et al., 2011). Therefore, the NS3-4A protease is a prime target for antiviral drug design.

NS3 protein consists of an N-terminal serine protease domain with its catalytic triad composed of His^{57}, Asp^{81} and Ser^{139}, and a C-terminal RNA helicase/NTPase domain. The NS3 helicase/NTPase couples NTP hydrolysis to unwind extensive RNA secondary structure and is important for RNA replication and virus assembly (Raney et al., 2010; Sharma, 2010).

The two domains of NS3 can function independently from each other, and the reason for their physical linkage remains unclear (Moradpour and Penin, 2013; Raney et al., 2010). The intracellular NS3 protease shows structure homology with extracellular serine proteases, but does not possess disulfide bonds to stabilize its structure as extracellular serine proteases (Abian et al., 2013). A Zn^{2+} ion together with its binding site formed by Cys^{97}, Cys^{99}, Cys^{145} and His^{149} stabilizes NS3 protease, activates NS3 hydrolysis, and facilitates NS2 processing at the NS2-NS3 junction. Binding of NS4A further stabilizes NS3 by restructuring the N-terminus of NS3 protease through the interaction with the central hydrophobic portion of NS4A, increases catalytic efficiency by influencing the spatial configuration of the catalytic triad, and directs the cellular membrane localization because of the high hydrophobicity of N-terminal transmembrane α-helix of NS4A. In addition, the C-terminal acidic portion of NS4A plays a role in regulating HCV genome replication and virus assembly by interacting with other viral proteins in the replication complex (Abian et al., 2010; Abian et al., 2013; Morikawa et al., 2011; Vega et al., 2013). NS4A also regulates HCV replication by modulating NS5A hyperphosphorylation (Lindenbach et al., 2007).

Liefhebber et al. has shown that NS3 might get phosphorylated in HCV subgenomic replicon (SGR) cells through phospho-specific staining and dephosphorylation assay. However,

phosphorylation efficiency is low and the phosphorylation sites are hard to identify. In addition, N-terminal acetylation of NS3 is identified by this research group through mass spectrometric analysis (Liefhebber et al., 2010). The role of NS3 phosphorylation and acetylation in the HCV life cycle needs to be further investigated.

It has been reported that protein arginine methyltransferases (PRMTs) can irreversibly and post-translationally methylate arginine residues in the arginine-glycine (RG)-rich region of many RNA-binding proteins (Gary and Clarke, 1998; Wolf, 2009). Since NS3 protein can bind to RNA through its RNA helicase domain and contains seven RG motifs including two RG motifs in the helicase domain, it is a potential methylation target for PRMTs. Full-length NS3 and NS3 helicase domain are shown to be methylated at Arg^{467} in the ^{460}Gln-Arg-Arg-Gly-Arg-Thr-Gly-Arg-Gly^{468} motif by PRMT1, but no methylation is found in NS3 protease domain (Rho et al., 2001). Mutation analysis has demonstrated that Arg^{464} and Arg^{467} are determinants for the helicase activity (Kim et al., 1997). Methylation of NS3 at Arg^{467} inactivates the helicase by inhibiting unwinding of double-stranded DNA (Duong et al., 2005). The reason that arginine methylation is involved in protein-nucleic acid interaction is that methyl modification may affect the binding affinity, protein stability, transcription and signal transduction (Gary and Clarke, 1998). Negative regulation of PRMT1 by protein phosphatase 2A (PP2A) increases NS3 helicase activity and enhances HCV RNA replication, therefore PP2A is considered as a potential target for HCV drug development (Duong et al., 2005).

The cofactor activity of NS4A is mediated by its central region and especially the hydrophobic Ile^{25} and Ile^{29} residues, since an I25A/I29A double mutant cannot form a complex with NS3 (Butkiewicz et al., 1996). To reactivate NS4A cofactor activity, the double mutant requires biotinylation at the N-terminus by biotin-aminohexanoic acid (Ahx). However, the N-terminal biotin fusion alone without Ahx or C-terminal biotin-Ahx fusion cannot restore NS4A cofactor activity. On the other hand, N-biotinylation of wild-type NS4A by biotin-Ahx can dramatically promote cofactor activity. Based on these data and the crystal structure, it is predicted that N-biotinylation by biotin-Ahx resembles a hydrophobic environment that enhances the stabilization of NS3-4A complex and C-biotinylation may sterically interfere with the substrate binding pocket (Butkiewicz et al., 2000).

1.1.5.8 The membranous web inducer NS4B

HCV NS4B is relatively poorly understood compared to other HCV proteins. The liberation of NS4B happens at last during HCV polyprotein precursor processing in a strictly defined position (Gouttenoire et al., 2010a). It is a 27 kDa highly hydrophobic integral membrane protein that induces the formation of the membranous web around the ER membrane where HCV genome replication takes place and functions by anchoring the HCV replication complex through an unknown mechanism (Choi et al., 2013; Sklan and Glenn, 2006). It has been reported that NS4B can interact with other viral proteins such as NS5A, bind viral RNA and has NTPase activity. It is involved in RNA replication, virus assembly and release (Einav et al., 2004; Jones et al., 2009). NS4B is also shown to activate ER stress pathways, contribute to steatosis by altering lipid metabolism, and escape from innate immune system by inhibiting interferon (Gouttenoire et al., 2010a). Moreover, its anti-apoptosis function might be associated with hepatocellular carcinoma development (Einav et al., 2008).

There are two amphipathic helices (AH1 and AH2) located in the N-terminal region of NS4B with their hydrophobic sides facing the cytoplasmic side of the ER membrane. AH2 is a membrane interacting domain that is essential for membrane trafficking, HCV genome replication and protein oligomerization. NS4B oligomerization is critical for replication complex formation (Gouttenoire et al., 2009; Gouttenoire et al., 2010b; Palomares-Jerez et al., 2013). The highly hydrophobic central core region of NS4B contains four transmembrane segments and the highly conserved C-terminus is a membrane binding domain that consists of two α-helical elements (H1 and H2) and plays a role in NS4B self-interaction, thus being important for replication complex formation (Liefhebber et al., 2009; Paul et al., 2011).

There are three common lipid modifications of protein located in lipid raft, including palmitoylation, N-terminal myristoylation and palmitoylation, and glycosylphosphatidylinositol modification (Melkonian et al., 1999). So far, only palmitoylation is detected in NS4B at Cys^{257} and Cys^{261} in the C-terminus and these two sites are relatively conserved among HCV genotypes. Site-directed mutagenesis confirmed that Cys^{261} palmitoylation is more crucial for protein-protein interaction and replication complex formation. Palmitoylation can enhance the polymerization activity of NS4B through its N-terminus (Yu et al., 2006).

1.1.5.9 The multi-functional NS5A

HCV NS5A is a phosphorylated zinc-metalloprotein without any enzymatic activity, but required for RNA replication and virion morphogenesis (Belda and Targett-Adams, 2012). However, the precise mechanism of how NS5A functions is not clear. It is demonstrated that NS5A can bind to HCV RNA, other HCV proteins such as NS5B and cellular proteins such as human vesicle-associated membrane-associated protein of 33 kDa (hVAP-33), thus contributing to replication complex formation (Chevaliez and Pawlotsky, 2006). It is also essential for the formation of double membrane vesicles (DMVs) where HCV replicates (Romero-Brey et al., 2015). In addition, NS5A has an inhibitory effect on HCV translation (Hoffman et al., 2015; Kalliampakou et al., 2005). Several other functions of NS5A include interferon resistance, transcriptional activation and signaling pathway regulation (Macdonald and Harris, 2004; Pawlotsky and Germanidis, 1999).

NS5A is composed of three domains that are linked by low-complexity sequences (LCSs). Domain I contains a zinc-binding motif and is the determinant for HCV RNA replication. It is a nucleic acid-binding domain that binds to the 3' G/C rich sequence in HCV RNA. It also functions in LD association and DMV induction. Domain II may play a role in evading innate immune response as well as RNA replication. Domain III participates in virus assembly and core protein interaction (Moradpour and Penin, 2013; Romero-Brey et al., 2015; Sharma, 2010; Suk-Fong Lok, 2013). In addition, there is an amphipathic α-helix in the N-terminal region responsible for ER membrane anchoring (Belda and Targett-Adams, 2012).

NS5A is a phosphoprotein that exists in two forms, basally phosphorylated form (56 kDa) and hyperphosphorylated form (58 kDa), which is conserved among HCV genotypes (Huang et al., 2007). The basally phosphorylated sites are mainly serine residues and the minority are threonine residues located in the central and C-terminal region. Major hyperphosphorylated sites are identified in a serine-rich region in the central portion of NS5A (Huang et al., 2007; Macdonald and Harris, 2004). The basally phosphorylated form may be affected by NS2 and NS4A, whereas hyperphosphorylation of NS5A requires NS3, NS4A and NS4B. Cellular protein kinases in the CMGC kinase family are involved in NS5A phosphorylation, including cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3) and CK2 (Asabe et al., 1997; Hijikata et al., 1993; Koch and Bartenschlager, 1999; Lindenbach et al., 2007; Macdonald and Harris, 2004). Since the subcellular distributions of both

NS5A forms are similar, the degree of phosphorylation does not affect NS5A localization to the ER membrane (Tanji et al., 1995). However, the degradation of NS5A is enhanced by increased degree of phosphorylation (Huang et al., 2007). Mutation analysis revealed that reduced NS5A hyperphosphorylation promotes HCV RNA replication, whereas reduced basal phosphorylation does not have an effect on HCV RNA replication in a replicon system. This suggests that the ratio of these two NS5A phosphorylation forms may be important for viral RNA replication (Appel et al., 2005; Neddermann et al., 2004). NS5A is also involved in virion production through its interaction with core protein, which requires basal phosphorylation of NS5A (Masaki et al., 2008). Therefore, NS5A may regulate HCV RNA replication and virion production by changing its phosphorylation profile.

NS5A is the only HCV protein that can be SUMOylated by covalently linking to small ubiquitin-related modifier (SUMO)-1 or -2 at NS5A Lys^{348}. NS5A SUMOylation stablizes NS5A through down-regulating ubiquitylation and is essential for its interaction with NS5B. Lee et al. also showed that silencing a key enzyme for SUMOylation or mutant Lys^{348} in HCV SGR RNA inhibits HCV replication, suggesting NS5A SUMOylation plays a role in HCV replication (Lee et al., 2014).

1.1.5.10 The RNA polymerase NS5B

HCV NS5B is a 68 kDa conserved RNA-dependent RNA polymerase (RdRp) that initiates complementary negative-strand RNA synthesis and then synthesizes positive-strand RNA using the newly synthetic negative-strand RNA as template. Due to the lack of proofreading of RdRp, HCV replication is error-prone (Moradpour and Penin, 2013; Sharma, 2010). NS5B can interact with other viral proteins such as NS3, NS4A and NS5A, and cellular proteins like hVAP-33, which facilitates the formation of RNA replication complex (Ishido et al., 1998; Tu et al., 1999). Furthermore, it can form a complex with the retinoblastoma tumor suppressor protein (pRb) and promote pRb degradation in a ubiquitin-dependent manner, therefore contributing to hepatocellular carcinoma development (Munakata et al., 2007).

Like other polymerases, the crystal structure of NS5B reveals that it resembles the configuration of a right hand. The finger, thumb and palm domains compose a unique shape. The active site located in the palm domain has a highly conserved GDD motif. There are four allosteric sites within the thumb and palm domains which serve as targets for antiviral

development (Beaulieu, 2009; Mayhoub, 2012; Membreno and Lawitz, 2011). Besides, NS5B is a tail-anchored protein with its C-terminal hydrophobic tail associated to the ER membrane (Sharma, 2010).

The function of many cellular enzymes for DNA and RNA metabolism and viral RdRps is often regulated by phosphorylation (Kim et al., 2004). Hwang et al. demonstrated that NS5B is a phosphoprotein in insect cells (Hwang et al., 1997). Kim et al. discovered that the protein kinase C-related kinase 2 (PRK2) is the specific enzyme for NS5B phosphorylation within the N-terminal finger domain. Knockdown and overexpression of PRK2 demonstrated PRK2 up-regulates HCV RNA replication in HCV SGR cells, suggesting that NS5B phosphorylation can enhance HCV replication (Kim et al., 2004).

Gao et al. identified an interaction between ubiquitin-like protein human homolog 1 of protein linking integrin-associated protein and cytoskeleton (hPLIC1) and NS5B (Gao et al., 2003). Since hPLIC1 interacts with both proteasome and E3 ubiquitin protein ligases E6AP and β-transducing repeat-containing protein (βTrCP), the ubiquitination modification of NS5B through hPLIC1 binding promotes ubiquitin-dependent proteasome degradation, resulting in decreased level of NS5B. NS5B mainly functions in RNA replication, so the decreased NS5B leads to HCV genome RNA reduction (Gao et al., 2003; Kleijnen et al., 2000). Although the ubiquitination sites within NS5B and the detailed mechanism of hPLIC1-induced NS5B degradation are still not clear, up-regulating NS5B ubiquitination may represent a target for antiviral development.

1.1.6 The HCV life cycle

1.1.6.1 HCV entry

The life cycle of HCV begins with virus attachment (Figures 1.2). Circulating HCV is often found as lipoviroparticles (LVPs) due to the association with low-density lipoproteins (LDLs) and very-low-density lipoproteins (VLDLs), so apolipoprotein (Apo) B and ApoE are also present in LVPs (Andre et al., 2002; Bartenschlager et al., 2011; Merz et al., 2011). The initial step of virus attachment happens between heparin sulfate proteoglycan (HSPG) on host cell surface and ApoE, HSPG and E2 (Barth et al., 2003; Jiang et al., 2012; Koutsoudakis et al., 2006). Cell surface low-density lipoprotein receptor (LDLR) that internalizes and clears circulating LDL

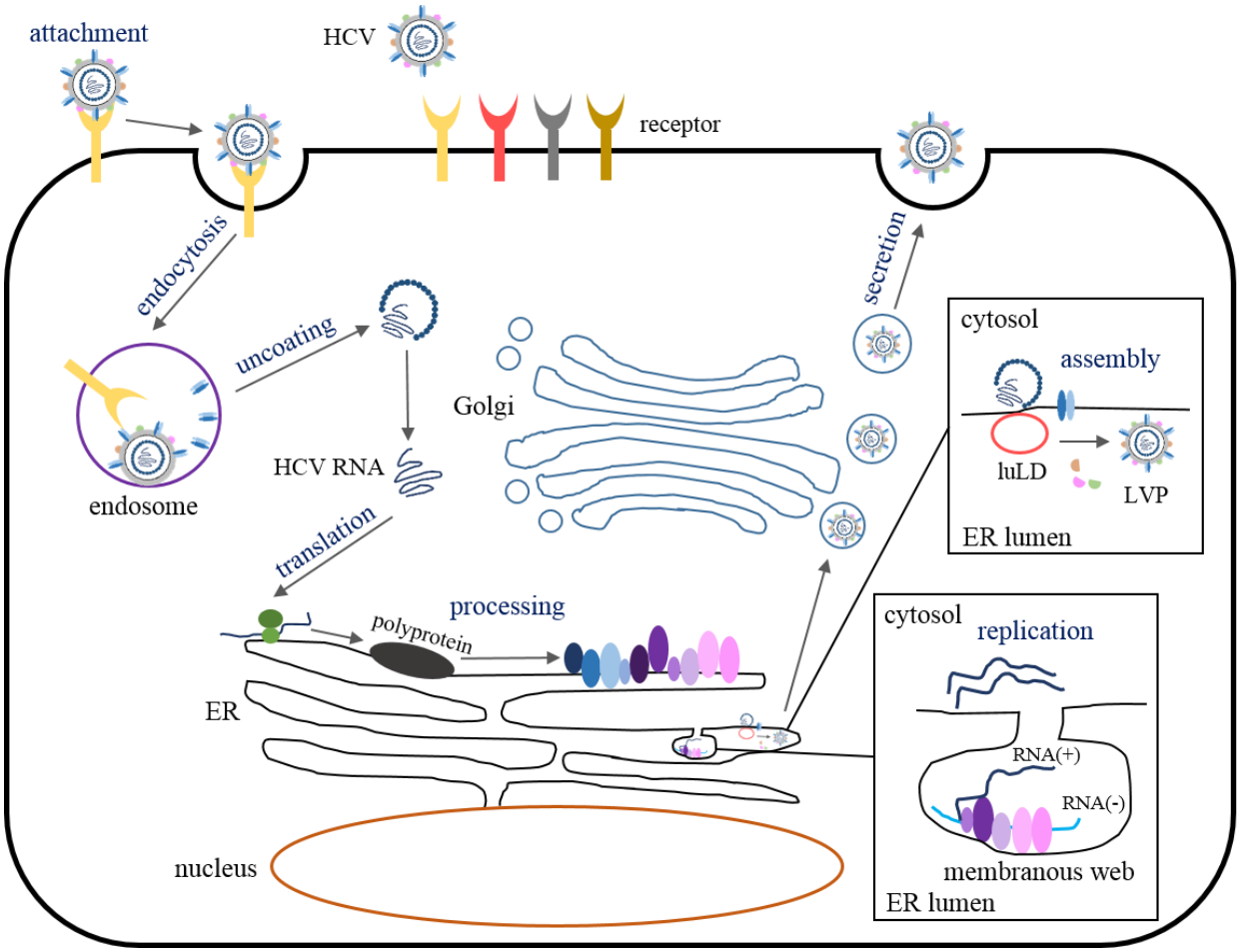

Figure 1.2 The HCV life cycle. The HCV life cycle is composed of virus attachment, endocytosis, uncoating, RNA translation, RNA replication, virion assembly and secretion.

can interact with LDL through ApoB and VLDL through ApoE (Hussain et al., 1999). Scavenger receptor class B type 1 (SR-B1) is another cell surface receptor involved in early virus attachment. It can also bind to ApoE and E2 (Dao Thi et al., 2012). The interaction between SR-B1 and E2 exposes CD81 binding sites in E2, so CD81 and E2 binding occurs at the late stage of virus attachment (Bankwitz et al., 2010; Dao Thi et al., 2012). The interaction between CD81 and E2 activates epidermal growth factor receptor (EGFR), thus EGFR downstream HRas is also activated. The process then triggers CD81 lateral diffusion, which can promote tight junction protein claudin-1 binding to LVP-CD81 (Brazzoli et al., 2008; Zona et al., 2013). Another tight junction protein occludin is also involved in HCV entry and acts after the formation of LVP-CD81-claudin-1 complex (Sourisseau et al., 2013). E1 cannot directly interact with cell surface receptors or molecules, but it affects the formation of E1-E2 heterodimer and the interaction between E2 and receptors (Wahid et al., 2013). The interactions between HCV and cell surface receptors or molecules result in rearrangement of receptors, LVP-associated lipoproteins and E1-E2 heterodimers. Then LVP-receptor complex induces inward budding and internalizes through clathrin-mediated endocytosis with clathrin-coated pits (Douam et al., 2015). Transferrin receptor (TfR) 1 that functions in iron uptake may play a role in the internalization step (Martin and Uprichard, 2013). The HCV-containing vesicles tether to and fuse with endosomes due to the acidic environment and the rearrangements caused by the interactions between E2 and SR-B1, E2 and CD81. The conformational change of E2 exposes putative E1 fusion peptide that can insert into host membrane. Sequentially, it facilitates lipid mixing and fusion of membranes. Finally, HCV genome is released into cytoplasm from viral nucleocapsid during the uncoating process (Douam et al., 2015). Cholesterol absorption receptor Niemann-Pick C1-like 1 (NPC1L1) is also involved in HCV entry since it can rearrange lipid on LVP after internalization (Sainz et al., 2012). Cell-to-cell transmission that can lead to escaping from neutralizing antibody is another pathway for HCV entry. It requires SR-B1, claudin-1, occludin and NPC1L1 (Barretto et al., 2014; Brimacombe et al., 2011; Timpe et al., 2008). It has been reported that E2 can interact with dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) or liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN). It suggests that HCV can infect other cells except for hepatocytes since DC-SIGN and L-SIGN express in dendritic cells (DCs) and liver endothelial cells, respectively (Lozach et al., 2003; Zeisel et al., 2011).

1.1.6.2 HCV RNA translation

HCV IRES-mediated viral translation initiates at the rough ER (Figure 1.2). IRES can bind to the small 40S ribosomal subunit via 5'UTR domain II, III and IV. It not only localizes the start codon at nucleotide position 342 close to ribosomal P-site by moving the start codon to mRNA binding cleft of the 40S ribosomal subunit, but also exposes the mRNA entry channel of the 40S ribosomal subunit through conformational change (Berry et al., 2011; Fraser and Doudna, 2007; Otto and Puglisi, 2004; Spahn et al., 2001). Then IRES-40S complex binds to eukaryotic initiation factor (eIF) 3 and ternary complex eIF2-GTP-Met-$tRNA_i^{Met}$ via 5'UTR domain III to form a 48S ribosomal complex. eIF3 stabilizes the ternary complex, while eIF2 delivers initiator tRNA to ribosomal P-site in the 40S ribosomal subunit (Hoffman and Liu, 2011; Niepmann, 2013; Otto and Puglisi, 2004). eIF5 binding to the 48S ribosomal complex via 5'UTR domain II hydrolyzes GTP and releases eIF2-GDP and eIF3, which facilitates start codon recognition. Following the large 60S ribosomal subunit association, translation-competent 80S ribosomal complex is formed. Finally, the 80S ribosomal complex proceeds along HCV genome and polyprotein is produced (Hellen, 2009; Locker et al., 2007; Lukavsky, 2009). Viral proteins are co- and post-translationally processed, which is described in detail in Chapter 1.1.5. The role of HCV 3'UTR in HCV translation is still unclear and researchers have shown different regulatory effects of the 3'UTR on HCV translation (Imbert et al., 2003; Murakami et al., 2001; Song et al., 2006). The 3'UTR can interact with multiple host and viral proteins as well as IRES, which leads to circularization of HCV RNA. It may regulate the switch between HCV translation and replication (Hoffman and Liu, 2011; Romero-Lopez et al., 2012; Song et al., 2006).

1.1.6.3 HCV RNA replication

After HCV RNA translation, viral proteins that are required for HCV replication including NS3, NS4A, NS4B, NS5A and NS5B are produced and compose HCV replication complex (Figure 1.2) (Gosert et al., 2003). HCV infection induces membrane rearrangement to form the membranous web where HCV replication complex is assembled. The membranous web containing DMVs and multiple membrane vesicles (MMVs) is derived by invaginating from the ER membrane. DMVs are the sites for HCV replication, while MMVs are not involved in HCV replication (Ferraris et al., 2010; Paul et al., 2013; Paul et al., 2014; Romero-Brey et al., 2012). NS5A induces the formation of DMVs although other HCV replication complex proteins can also

alter membrane morphogenesis. NS4B is a transmembrane protein and its oligomerization through cytoplasmic AH2 and C-terminus provides scaffold for membrane vesicles (Egger et al., 2002; Paul et al., 2011; Romero-Brey et al., 2015; Romero-Brey et al., 2012). In the membranous web, NS5B binds to the 3'UTR and initiates the synthesis of negative-strand RNA. The newly synthesized negative-strand RNA serves as template for mass production of positive-strand RNA through *de novo* initiation of RNA synthesis (Lohmann, 2013; Paul et al., 2014).

1.1.6.4 HCV virion assembly and secretion

HCV virion assembly and secretion are not fully understood (Figure 1.2). Mature core can attach to the surface of LDs, which elevates perinuclear LD level. The interaction between core and NS5A makes NS5A also localize on the surface of LDs and probably transports newly synthesized HCV RNA from the membranous web to core (Boulant et al., 2008; McLauchlan, 2000, 2009). The interaction between NS2 and p7 brings E1-E2 heterodimer and NS3-4A to assembly site on the ER membrane. The sequential interaction between NS2-p7 and NS3-4A recruits LD-associated core to assembly site on the ER membrane. Then nucleocapsid forms at the ER surface and buds into the ER lumen, but how HCV RNA is encapsidated is not known (Counihan et al., 2011; Lindenbach, 2013). In the ER lumen, glycoproteins are incorporated onto the nucleocapsid. The viral particles then fuse with luminal lipid droplets (luLDs) that are VLDL precursors to form LVPs which are associated with ApoB and ApoE. LVPs are exported via VLDL secretion pathway. Endosomal trafficking is involved in the release of HCV virion and p7 protects HCV virion from the acidic compartments (Coller et al., 2012; Paul et al., 2014; Wozniak et al., 2010).

1.1.7 HCV treatment and prevention

1.1.7.1 Traditional HCV treatment

The traditional standard therapy is a dual therapy that uses pegylated interferon α (PEG-IFNα)-2a or -2b and ribavirin. Pegylation of interferon (IFN) α through covalently linking polyethylene glycol (PEG) to IFNα can increase IFNα half-life and reduce IFN injection frequence (Parekh and Shiffman, 2014; Tsubota et al., 2011). The oral antiviral ribavirin is a guanosine analog that can increase mutation rate during viral replication and inhibit NS5B polymerase. It also plays an immunomodulatory role and works synergistically with IFNα (Jain

and Zoellner, 2010). The definition of sustained virological response (SVR) is undetectable serum HCV RNA 24 weeks after antiviral treatment and the SVR rate is a measurement of therapy efficacy. The SVR rate of the dual therapy is 40-50% in HCV genotype 1 infected patients which dominate in HCV-infected population, 75-90% in HCV genotype 2 or 3 infected patients and 65-85% in HCV genotype 4, 5, or 6 infected patients. Several factors are also associated with SVR rate: host factors (e.g., age, obesity, diabetes, ALT level, presence of cirrhosis or steatosis, and IL-28B gene polymorphisms), viral factors (e.g., HCV genotype, baseline serum HCV RNA level and mutation in core or NS5A), and treatment-related factors (e.g., dose and treatment duration) (Hofmann and Zeuzem, 2011; Sulkowski et al., 2011; Tsubota et al., 2011; Zhu and Chen, 2013). However, the dual therapy may induce severe adverse effects, including anemia, neutropenia, thrombocytopenia, cardiovascular diseases, autoimmune disorders and depression, etc. It results in discontinuing treatment in 10-15% of patients (Cheng et al., 2015). Beside, in difficult-to-treat patients who have cirrhosis, organ transplant, renal diseases or HCV/HIV co-infection, the use of the dual therapy is limited (Ghany et al., 2009).

1.1.7.2 Direct-acting antivirals

In the past few years, a number of direct-acting antivirals (DAAs) that target HCV nonstructural proteins have been approved by Food and Drug Administration (FDA). They can be divided into four categories: NS3-4A protease inhibitor, NS5A inhibitor, NS5B polymerase inhibitor and combination tablet of nonstructural protein inhibitors (Figure 1.1). They can reduce the treatment time and significantly improve cure rate, but they are high costs.

NS3-4A protease inhibitors prevent HCV proprotein processing, which leads to reduced production of nonstructural proteins and disrupts the HCV life cycle (Conteduca et al., 2014). In 2011, the first two DAAs, Incivek (telaprevir) and Victrelis (boceprevir), which are oral NS3-4A protease inhibitors, were approved for clinical treatment. They are administered in combination with PEG-IFNα and ribavirin as triple therapy mostly used in chronic HCV genotype 1 infection patients (Pawlotsky, 2013; Sarrazin et al., 2012). The triple therapy significantly improves efficacy by increasing SVR rate compared to traditional standard therapy (Assis and Lim, 2012). However, the limitations of these two DAAs include being delivered every eight hours, adverse effects and the possibility of viral breakthrough (Kim et al., 2012; Lam and Jacob, 2012). Due to the approval of the more effective DAAs, telaprevir and boceprevir were discontinued in 2015

(Zhang, 2016). Another oral NS3-4A protease inhibitor, Olysio (simeprevir), was approved in 2013. It is used in combination with PEG-IFNα and ribavirin to treat HCV genotype 1 or 4 infection (Sanford, 2015; Vaidya and Perry, 2013). Compared to telaprevir and boceprevir, it is delivered daily, and has less adverse effects and no reported drug-drug interactions (You and Pockros, 2013).

Sovaldi (sofosbuvir), approved in 2013, is an oral pan-genotypic NS5B polymerase inhibitor that is unlikely for viral breakthrough and used to treat HCV genotype 1 to 4 infection. It is a uridine analog that mimics and competes with polymerase substrates and thus terminates HCV replication. It is administered in combination with PEG-IFNα and ribavirin to treat HCV genotype 1 or 4 patients. However, it can be used in IFN-free treatment with ribavirin in HCV genotype 2 or 3 patients. Sofosbuvir can also be used in HCV patients waiting for liver transplant or HCV/HIV co-infected patients (Chopp et al., 2015; Conteduca et al., 2014; Keating, 2014; Keating and Vaidya, 2014).

Daklinza (daclatasvir), approved in 2015, is an oral NS5A inhibitor with suboptimal genetic barrier to resistance. It not only inhibits polyprotein processing by NS3-4A and NS5A hyperphosphorylation, but also interferes with the formation of replication complex and virion assembly. It is used with sofosbuvir to treat HCV genotype 1 or 4 infection, and with sofosbuvir and ribavirin to treat HCV genotype 3 infection. For HCV genotype 4 infection, daclatasvir can also be used in combination with PEG-IFNα and ribavirin as triple therapy to increase SVR rate. Besides, it is also used in cirrhosis, liver transplant or HCV/HIV co-infected patients (Jafri and Gordon, 2015; Manolakopoulos et al., 2016; McCormack, 2015).

Several oral combination tablets of nonstructural protein inhibitors have been approved by FDA recently. They can be administered in IFN-free treatment and achieve the SVR rate of 90-100% according to HCV genotype. Harvoni, approved in 2014, is composed of ledipasvir (NS5A inhibitor) and sofosbuvir. It is used in HCV genotype 1, 3, 4, 5 or 6 infection, or HCV/HIV co-infection. For HCV genotype 1, 3 or 4 infection with cirrhosis or liver transplant, it is used in combination with ribavirin (Bourliere et al., 2015; Keating, 2015). Viekira Pak, approved in 2014, is composed of paritaprevir (NS3-4A protease inhibitor), ritonavir (paritaprevir pharmacologic booster), ombitasvir (NS5A inhibitor) and dasabuvir (non-nucleotide analog NS5B inhibitor). It is used in HCV genotype 1 infection, and ribavirin is also used in combination in cirrhosis, liver transplant or HCV/HIV co-infected patients (Cheng et al., 2015; Deeks, 2015). Technivie,

approved in 2015, is composed of paritaprevir, ritonavir and ombitasvir. In combination with ribavirin, it is used to treat HCV genotype 4 patients (Keating, 2016). Zepatier, approved in 2016, is composed of elbasvir (NS5A inhibitor) and grazoprevir (NS3-4A protease inhibitor). It is used with or without ribavirin in HCV genotype 1 or 4 infection, or patients with cirrhosis, HCV/HIV co-infection. It is also effective in difficult-to-treat patients that have inherited blood disorders or advanced chronic kidney diseases (Carrion and Martin, 2016; El Kassas et al., 2016). Epclusa, approved in 2016, is composed of velpatasvir (NS5A inhibitor) and sofosbuvir. It is the first DAA that can treat all HCV genotype infection, and also used in cirrhosis, HCV/HIV co-infected, or renal impaired patients (Miller, 2017). Vosevi (sofosbuvir/velpatasvir/voxilaprevir (NS3-4A protease inhibitor)) and Mavyret (glecaprevir (NS3-4A protease inhibitor)/pibrentasvir (NS5A inhibitor)), approved in 2017, can be used in all HCV genotype infection or certain previous treatment failed patients (Abutaleb et al., 2018; Heo and Deeks, 2018).

1.1.7.3 HCV vaccine

HCV has been identified for almost 30 year, but no HCV vaccine is available. There are several reasons that make HCV vaccine development challenging. Firstly, there are limited practicable animal models since chimpanzee is the only primate that can be infected with HCV except for human (Burm et al., 2018). Secondly, RdRp NS5B lacks proofreading, which contributes to HCV genetic variability. The neutralizing antibodies induced by certain HCV strain are not pan-genotypic. Thirdly, the association with lipoproteins of HCV and glycosylation of HCV envelope proteins mask the epitopes and shield HCV from neutralizing antibodies (Cashman et al., 2014; Fauvelle et al., 2016; Helle et al., 2011; Voisset et al., 2006). Fourthly, the cell-to-cell transmission does not require HCV virion to present in serum, which results in the escape of HCV from neutralizing antibodies (Brimacombe et al., 2011). Fifthly, mutations in CD8+ T cell epitopes lead to viral escape from T cell responses. Sixthly, chronic HCV infection makes HCV-specific T cell dysfunctional and inhibits T cell responses through regulatory T cells (Neumann-Haefelin and Thimme, 2013). An ideal HCV vaccine should protect from a variety of HCV strains with robust and prolonged immune responses.

HCV vaccines are still in clinical development phase and can be divided into following categories: recombinant viral vector vaccine (e.g., expressing structural or nonstructural proteins in adenovirus), peptide vaccine (chemically synthesized immunogenic peptides), recombinant

protein subunit vaccine (e.g., recombinant E1 and E2), DNA vaccine (e.g., core gene) and DC-based vaccine (stimulated DCs infused with HCV antigens) (Abdelwahab and Ahmed Said, 2016; Naderi et al., 2014). For prophylactic vaccine trials, E1-E2 or NS3-NS5B is used as antigen. For therapeutic vaccine trials, NS3-NS5B, NS3-4A, NS3-core fusion protein, or five synthetic peptides from core, NS3 and NS4 are used as antigen (Li et al., 2015).

1.2 Proprotein Convertase Subtilisin/Kexin Type 9

1.2.1 Proprotein convertases

Proprotein convertases (PCs) are secretory serine proteases that are responsible for post-translational proteolysis of inactive secretory precursors into bioactive mature proteins. There are nine members, including PC1/3, PC2, furin, PC4, PC5/6, paired basic amino acid cleaving enzyme 4 (PACE4), PC7, subtilisin/kexin isozyme (SKI)-1/site 1 protease (S1P) and proprotein convertase subtilisin/kexin type 9 (PCSK9) (Seidah and Prat, 2012; Seidah et al., 2013). The first seven PC members cleave precursors after single or paired basic residues in the sequence of Lys/Arg-$X_{0,2,4,6}$- Lys/Arg↓. The cleavage sites for the last two PC members are not basic amino acid-specific. SKI-1/S1P cleaves precursors after the sequence of Arg-X-Leu/Val/Ile-X↓ (where X cannot be cysteine or proline) and PCSK9 only cleaves itself after the sequence of Val-Phe-Ala-Gln↓ (Seidah, 2011a; Seidah and Prat, 2012). PCs have high degree of homology and the structure similarity is that they all have a signal peptide (SP), a prodomain (PD) and a catalytic domain (CA) at the N-terminus. PC2 activation by PD removal requires the binding protein 7B2, while the activation of other PCs depends on post-translationally auto-catalytic cleavage of PD (Seidah et al., 1999; Seidah and Prat, 2012). PC1/3 and PC2 are primarily expressed in nervous system and endocrine tissues, and they can sometimes compensate for each other. The substrates of PC1/3 function in food ingestion and energy homeostasis, thus PC1/3 deficiency causes endocrine diseases including obesity and diabetes. The substrates of PC2 are proneuropeptides and secretory pathway propeptides, so PC2 plays a role in growth and metabolism (Ramos-Molina et al., 2016; Seidah et al., 2013; Stijnen et al., 2016; Zhang et al., 2010). PC4 is expressed in testis and ovary, and involved in reproduction by regulating fertility and gestation (Seidah, 2011b). Furin, PC5/6 and PACE4 are widely distributed and target constitutively secreted substrates. Furin has wider substrates compared to PC5/6 and PACE4, and functions in embryogenesis, development, homeostasis, neurodegeneration, tumor metastasis and pathogen

virulence. PC5/6 and PACE4 can control early embryonic development, axis formation, bone morphogenesis and tumorigenesis, and have strong redundancy with furin (Bassi et al., 2000; Seidah et al., 2008; Seidah and Prat, 2012; Thomas, 2002). PC7 and SKI-1/S1P are also ubiquitously expressed. PC7 can regulate iron metabolism, mood modification and tumorigenesis, and sometimes plays a redundant role with furin. SKI-1/S1P is involved in cholesterol and fatty acid synthesis, cartilage formation and viral virulence (Seidah, 2011a; Seidah et al., 2013). PCSK9 is the last member discovered in the PC family in 2003. It is primarily expressed in the liver, but can also be found in many other tissues, including intestine, kidney, pancreas, adipocytes, central nervous system and vascular wall (Mombelli et al., 2015; Norata et al., 2014; Seidah et al., 2014). The major function of PCSK9 is to post-translationally down-regulate LDLR by enhancing the degradation of LDLR, which results in elevated low-density lipoprotein cholesterol (LDLC) in plasma. Therefore, it is a risk factor for familial autosomal dominant hypercholesterolemia (Lambert et al., 2009; Tibolla et al., 2011).

1.2.2 PCSK9 structure and maturation

PCSK9 gene is localized in human chromosome 1p32 and its protein consists of a small SP (aa. 1-30), a PD (aa. 31-152), a CA (aa. 153-421), a small hinge region (HR) (aa. 422-439) and a C-terminal cysteine- and histidine-rich domain (CHRD) (aa. 440-692) containing three tightly packed modules (CM1, CM2 and CM3) (Du et al., 2011; Marais et al., 2012; Seidah et al., 2014). Du et al. studied the role of different domains using truncated PCSK9. PD deletion completely abolishes PCSK9 secretion and LDLR degradation ability. CHRD deletion does not affect auto-cleavage or secretion, and only impacts LDLR degradation. CHRD alone can be secreted and interact with LDLR, but loses LDLR degradation ability (Du et al., 2011). CA can interact with LDLR, but its catalytic activity is not related to LDLR degradation (Li et al., 2007).

Signal peptidase can co-translationally cleave the SP from the newly synthesized preproPCSK9 to produce proPCSK9 (72 kDa) in the ER. PD is an intramolecular chaperone that guides proPCSK9 to fold into an active confirmation. After PD is auto-cleaved at Gln^{152}, mature PCSK9 (63 kDa) is formed. This step plays a vital role in PCSK9 maturation and secretion. However, unlike other PCs, the cleaved PD and mature PCSK9 form a PCSK9-PD complex non-covalently linked by hydrogen bonds. Then the complex is transported to Golgi and secreted through secretory pathway (Farnier, 2014; Lambert et al., 2009; Poirier and Mayer, 2013; Seidah

and Prat, 2012; Seidah et al., 2013). However, the secreted complex is enzymatically inactive because the attached PD inhibits the catalytic activity by blocking substrates to access catalytic triad, which implies that PCSK9 has no other substrate besides itself and its catalytic activity is not required for PCSK9-induced LDLR degradation. Mutation of catalytic triad residues results in PCSK9 inactivation and degradation (Cunningham et al., 2007; Piper et al., 2007).

Another truncated form of PCSK9 (aa. 219-692) (55 kDa) has also been detected in human plasma. It is produced through the cleavage at Arg^{218} by other PC family members such as furin and PC5/6A, and its ability of LDLR down-regulation is reduced since a portion of the LDLR-interacting domain is cleaved (Benjannet et al., 2006; Lipari et al., 2012).

1.2.3 Regulation of PCSK9

At transcriptional level, PCSK9 gene expression is regulated by intracellular cholesterol concentration via sterol-regulatory element binding proteins (SREBPs) (Norata et al., 2014). There is sterol-regulatory element (SRE), hepatocyte nuclear factor (HNF) binding site, forkhead box O3 (FoxO3) binding site and specificity protein 1 (Sp1) motifs in the PCSK9 promoter region (Chen et al., 2016; Jeong et al., 2008). So PCSK9 expression can be promoted by transcription factors such as SREBP-1a, SREBP-1c, SREBP-2, HNF-1α and Sp1 (Costet et al., 2006; Costet et al., 2008; Jeong et al., 2008; Li et al., 2009). Several nuclear receptors also show modulating roles in PCSK9 promoter. Farnesoid X receptor (FXR) induces a reduction in PCSK9 expression (Langhi et al., 2008), while liver X receptor (LXR) induces an increase in PCSK9 expression (Costet et al., 2006). Peroxisome proliferator-activated receptor (PPAR) α differently regulates PCSK9 expression based on different studies. PPARγ activation promotes PCSK9 expression (Duan et al., 2012). In addition, PCSK9 expression can be epigenetically regulated. It has been reported that histone nuclear factor P (HINFP), its cofactor nuclear protein of the ataxia telangiectasia mutated locus (NPAT), and histone acetyltransferase (HAT) cofactor transformation/transactivation domain-associated protein (TRRAP) recruited by NPAT can mediate histone H4 acetylation on PCSK9 promoter, thus up-regulating PCSK9 expression (Li and Liu, 2012). Tao et al. has demonstrated that histone deacetylase (HDAC) sirtuin6 recruited by FoxO3 can suppress PCSK9 gene expression by deacetylating histone H3 on PCSK9 promoter (Tao et al., 2013).

At post-transcriptional level, mRNA stability and translation are involved in regulating PCSK9 expression. It has been reported that the high AU content in the 3'UTR of mRNA is associated with instability of mRNA (Wu and Brewer, 2012). There are two AUUUA AU-rich sequences located in the extreme end of PCSK9 mRNA 3'UTR, indicating PCSK9 expression may be repressed by its 3'UTR (Seidah et al., 2014). Besides, PPARα can up-regulate the expression of furin and PC5/6A (Kourimate et al., 2008), and mature PCSK9 can be cleaved at Arg^{218} by furin and PC5/6A to produce a 55 kDa truncated form (Benjannet et al., 2006). It reduces functional PCSK9 in plasma.

1.2.4 PCSK9 function

1.2.4.1 PCSK9, LDLR degradation and lipoprotein regulation

Normally, circulating LDL targets to ligand-binding domain of LDLR and is internalized together via clathrin-mediated endocytosis. After LDL is detached in the acidic sorting endosome, ligand-binding domain folds back and LDLR presents a closed conformation that is essential for its recycling. So LDLR is subsequently recycled back to the plasma membrane for continuous binding of LDL (Figure 1.3) (Leren, 2014; Rudenko et al., 2002). Secreted PCSK9 can bind to the extracellular epidermal growth factor (EGF) repeat A in the EGF homology domain of LDLR primarily via its CA and is endocytosed together with LDLR through clathrin-coated pits (Zhang et al., 2007). In the acidic sorting endosome, a second step binding occurs between PCSK9 CHRD and LDLR ligand-binding domain (Yamamoto et al., 2011). The binding affinity between PCSK9 and LDLR is dramatically enhanced, thus the receptors are locked in an open conformation. The extended conformation of LDLR prevents it from recycling to cell surface, thereby promoting lysosomal degradation (Figure 1.3) (Duff and Hooper, 2011; Lambert et al., 2012; Marais et al., 2012). Intracellular PCSK9 can also promote LDLR intracellular degradation, which probably initiates in post-ER compartment before LDLR transporting to cell surface (Figure 1.3) (Maxwell et al., 2005; Schulz et al., 2015). Gain-of-function PCSK9 mutants increase LDLR degradation, which up-regulates circulating LDLC. They can lead to the development of hypercholesterolemia, atherosclerosis and cardiovascular diseases (Schulz et al., 2015).

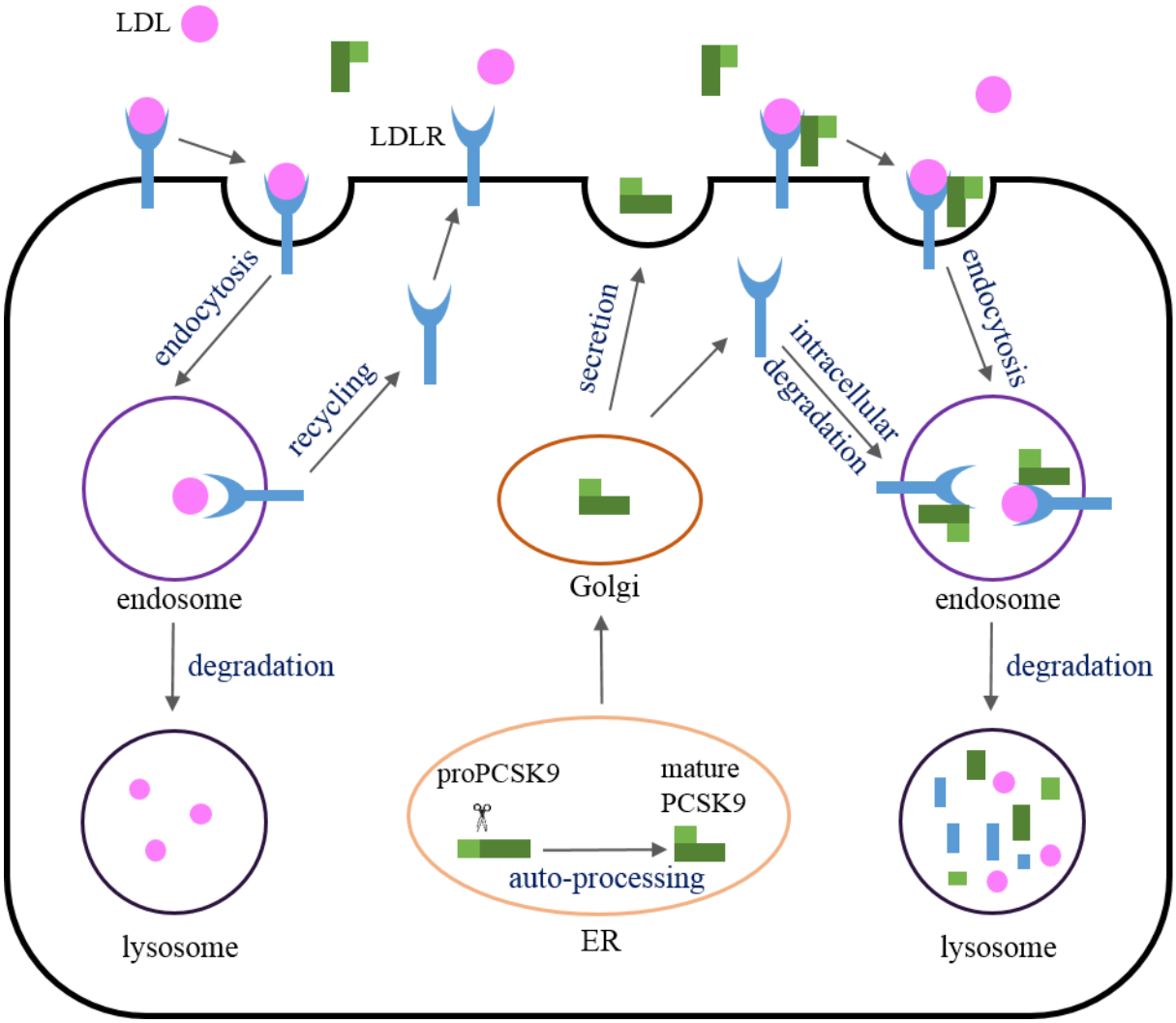

Figure 1.3 PCSK9-mediated LDLR degradation. Normally, LDL targets to LDLR and is internalized together via clathrin-mediated endocytosis. After LDL is detached in the acidic endosome, LDLR is recycled back to the plasma membrane. Secreted PCSK9 can bind to LDLR and is endocytosed together with LDLR. The binding affinity between PCSK9 and LDLR is enhanced in the acidic environment, thus the receptors are locked in an open conformation. It prevents LDLR from recycling to cell surface, thereby promoting lysosomal degradation. Intracellular PCSK9 can also promote LDLR intracellular degradation.

It is found that PCSK9 can bind to ApoB-containing lipoprotein LDL in plasma, which results in increased ApoB production by preventing ApoB degradation and suppressed LDLR degradation by reducing PCSK9-LDLR binding (Kosenko et al., 2013; Sun et al., 2012). This interaction mainly happens with mature PCSK9 (63 kDa), but not with truncated PCSK9 (55 kDa) (Han et al., 2014). PCSK9 can also interact with and down-regulate VLDLR, apolipoprotein E receptor 2 (ApoER2) and CD36. It is associated with plasma triglyceride (TG) level through increasing plasma TG-rich lipoprotein which can be down-regulated by LDLR, VLDLR, ApoER2 and CD36. So PCSK9 can reduce fatty acid uptake, TG accumulation in tissues and the risk of visceral adiposity (Blasiole et al., 2008; Costet et al., 2006; Lakoski et al., 2009; Moore and Goldberg, 2016; Poirier et al., 2008; Roubtsova et al., 2011). In addition, PCSK9 up-regulates lipogenesis via not only promoting SREBP-1c-fatty acid synthase (FAS) pathway, but also increasing the production of non-SRE-regulated ApoB, ApoE, diacylglycerol acyltransferase 1 (DGAT1) and microsomal triglyceride transfer protein (MTTP) (Norata et al., 2016; Tavori et al., 2016). These may all lead to dyslipidemia.

1.2.4.2 PCSK9 and viral infection

Labonte et al. were the first to demonstrate that PCSK9 could inhibit HCV infection *in vitro* and hypothesize plasma PCSK9 level correlates with HCV infectivity (Labonte et al., 2009). They identified that PCSK9 expression markedly decreases cell surface LDLR and CD81 in Huh-7 cells, but not cell surface claudin 1. CD81 expression reduction upon PCSK9 is independent of LDLR level in Huh-7 cells, which is further confirmed *in vivo* using PCSK9 single knockout and PCSK9 and LDLR double knockout mice. However, SR-B1 is not affected in PCSK9 knockout or PCSK9-overexpressing mice compared to wild type. They also showed that PCSK9 impedes HCV genotype 2a infection in PCSK9-overexpressing and soluble PCSK9-treated Huh-7 cells. In PCSK9-overexpressing Huh-7 cells, HCVpp entry is inhibited, which is probably related to CD81 degradation (Labonte et al., 2009). A contradictory result that CD81 expression is not significantly affected by PCSK9 in Huh-7 cells was shown by Mazumdar et al. They proved that wild-type and gain-of-function mutant PCSK9 overexpression reduce HCVpp-E1-E2 and HCVpp-E1 infectivity, but not HCVpp-E2 infectivity, implying that HCVpp-E1 entry is LDLR dependent while HCVpp-E2 entry is LDLR independent (Mazumdar et al., 2011). A recent published paper determined that PCSK9 partially co-localizes with cell surface LDLR and CD81,

and down-regulates both cell surface and total expression of LDLR and CD81 through PCSK9 CA and CHRD in Huh-7 cells. Similar to LDLR, PCSK9 can interact with CD81 and down-regulate CD81 expression via intracellular pathway. They also demonstrated that CD81 down-regulation by PCSK9 and the interaction between PCSK9 and CD81 are independent of LDLR using CHO-A7 cells that constitutively lack LDLR, but LDLR expression can further promote CD81 degradation by PCSK9 (Le et al., 2015). It has been published that PCSK9 inhibits HCV replication in both HCV-2a replicon cells and HCV-2a-infected Huh-7 cells. Compared to wild type, gain-of-function mutant PCSK9 induces slightly more inhibitory effect on HCV replication (Syed et al., 2014).

1.2.4.3 Other PCSK9 functions

Except for the liver, PCSK9 is expressed and functions in other tissues. In the intestine, PCSK9 can enhance LDLR degradation and lipogenesis, thus promoting TG-rich lipoprotein expression and lipid accumulation in enterocytes (Rashid et al., 2014). It also down-regulates NPC1L1, which results in decreased intestinal cholesterol absorption (Levy et al., 2013). In the kidney, epithelial Na^+ channel (ENaC) that increases Na^+ re-absorption from urine can interact with PCSK9. The interaction promotes the degradation of ENaC and reduces cell surface ENaC, which may reduce the risk of hypertension (Sharotri et al., 2012). Elevated plasma PCSK9 level is associated with renal diseases, such as proteinuria and chronic kidney disease (Jin et al., 2014; Konarzewski et al., 2014; Kwakernaak et al., 2013). It suggests that PCSK9 may worsen renal diseases or renal diseases may lead to dyslipidemia. In the pancreas, PCSK9 deficiency results in pancreatic dysfunction, including glucose intolerance, decreased insulin secretion, increased diabetes incidence, and morphology change of pancreatic islets (Mbikay et al., 2010; Saavedra et al., 2015). In the brain, PCSK9 plays a role in neurogenesis (Norata et al., 2016), and VLDLR and ApoER2 that are PCSK9 targets are involved in cerebellar development (Trommsdorff et al., 1999). PCSK9 can also up-regulate neuronal apoptosis by increasing caspase-3 activity and c-Jun phosphorylation, and decreasing ApoER2 (Kysenius et al., 2012; Norata et al., 2016). Besides, it is related to neurocognitive dysfunction. On one hand, hypercholesterolemia caused by PCSK9-mediated LDLR degradation can lead to neurodegenerative diseases including Alzheimer's disease (AD) (Xue-Shan et al., 2016). On the other hand, PCSK9 can reduce amyloid β peptide production by down-regulating β-site amyloid precursor protein-cleaving enzyme 1 (BACE1),

thus preventing AD development (Jonas et al., 2008; Obulesu and Lakshmi, 2014). In the vascular wall, PCSK9 decreases LDLR in macrophages, which accumulates lipid in the artery wall and facilitates atherosclerosis progression (Ferri et al., 2012; Linton et al., 1999). PCSK9 mediated-inflammatory response is also involved in atherosclerotic lesion (Norata et al., 2016).

1.2.5 PCSK9 inhibitors

Considering that PCSK9 is associated with plasma LDLC level, PCSK9 inhibitors are therapeutic candidates for some lipid disorders. In 2015, the first two monoclonal PCSK9 antibodies were approved by FDA. Praluent (alirocumab) and Repatha (evolocumab) are used in adults with familial hypercholesterolemia or clinical atherosclerotic cardiovascular disease whose LDLC levels need to be further reduced even after diet control and statin or other lipid-lowering treatments (Chaudhary et al., 2017). They can prevent PCSK9 binding to LDLR and increase LDLR on cell surface, which significantly facilitates LDLC uptake and results in 50-60% of circulating LDLC reduction. Besides, they only cause minor adverse reactions that include nasopharyngitis, influenza, injection site reactions and allergic reactions (Farnier, 2015; Raedler, 2016; Wiggins et al., 2018). Other monoclonal PCSK9 antibodies are in clinical trail phase. Non-antibody approaches to inhibit PCSK9, such as PCSK9 vaccine, LDLR mimic peptides, gene silencing and auto-processing inhibition, are still in development (Chaudhary et al., 2017; Glerup et al., 2017; Yadav et al., 2016).

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Rationale

HCV can differently modulate transcription factors involved in PCSK9 expression including SREBPs, HNF-1 and FoxO3 (Bose et al., 2014; Li et al., 2009; Matsui et al., 2012; Qadri et al., 2006; Tao et al., 2013; Waris et al., 2007), so HCV may affect PCSK9 expression via regulating these transcription factors. On the other hand, PCSK9 can regulate lipid levels (Norata et al., 2016) and the HCV life cycle is closely connected with lipid metabolism (Popescu et al., 2014; Schaefer and Chung, 2013), so PCSK9 may regulate different stages of the HCV life cycle. These observations suggest that there may be complex interactions between HCV and PCSK9.

2.2 Hypothesis

HCV can regulate PCSK9 expression, which in turn impacts different stages of the HCV life cycle.

2.3 Objectives

Objective 1: Study the role of PCSK9 in different stages of the HCV life cycle

(1) Identify the effect of PCSK9 on HCV translation, replication, assembly and secretion
(2) Identify the mechanism if PCSK9 affects certain stage of the HCV life cycle

Objective 2: Determine the effect of HCV on PCSK9 expression

3.0 PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9 INHIBITS HEPATITIS C VIRUS REPLICATION THROUGH INTERACTING WITH NA5A

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Keywords: PCSK9; HCV replication; NS5A; Protein interaction; NS5A dimerization; RNA binding

Running title: **PCSK9 inhibits HCV replication**

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3.2 Authors' Contribution

All the experiments within this chapter were performed by Zhubing Li. The manuscript was written by Zhubing Li and edited by Qiang Liu.

3.3 Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease actively involved in regulating lipid homeostasis. Although PCSK9 has been shown to inhibit hepatitis C virus (HCV) entry and replication, the underlying mechanisms have not been thoroughly characterized. Moreover, whether PCSK9 regulates HCV translation and assembly/secretion has not been determined. We therefore further studied the effects of PCSK9 on the HCV life cycle. We showed that PCSK9 did not affect HCV translation or assembly/secretion. Overexpression of PCSK9 inhibited HCV replication in HCV genomic replicon cells in a dose-dependent manner and after cell culture-derived HCV (HCVcc) infection. Knocking down PCSK9 increased HCV replication. The gain-of-function (D374Y) or loss-of-function (Δaa. 31-52) PCSK9 mutants for low-density lipoprotein receptor (LDLR) degradation had no effect on HCV replication, suggesting that HCV replication inhibition by PCSK9 was not due to LDLR degradation. The uncleaved proPCSK9, but not cleaved PCSK9, down-regulated HCV replication, suggesting that the auto-cleavage of PCSK9 affected HCV replication. We also found that PCSK9 interacted with NS5A through NS5A aa. 95-215, and this region played an important role in NS5A dimerization, NS5A-RNA binding and was essential for HCV replication. More importantly,

NS5A dimerization and NS5A-RNA binding were suppressed by PCSK9 upon interaction. These results suggested that PCSK9 inhibited HCV replication through interaction with NS5A. Our study should help optimize anti-HCV treatment regimen in patients with abnormal lipid profiles.

3.4 Introduction

Hepatitis C virus (HCV), which belongs to the genus *Hepacivirus* in the *Flaviviridae* family, is an enveloped positive-sense single-stranded RNA virus with seven major genotypes (Ashfaq et al., 2011; Smith et al., 2014). Its genome is approximately 9.6 kb and encodes a polyprotein precursor that can be cleaved into three structural proteins core, envelope glycoproteins E1 and E2, and seven nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Moradpour and Penin, 2013; Suzuki et al., 2007). HCV infects a population of about 170 million worldwide and 70%-80% of exposed individuals develop into chronic infection (Lauer, 2013; Westbrook and Dusheiko, 2014). Chronic HCV infection can result in progressive liver damage, including steatosis, cirrhosis and hepatocellular carcinoma, which eventually leads to liver failure (Maasoumy and Wedemeyer, 2012). Although recently approved direct-acting antivirals have significantly improved efficacy and safety compared to traditional interferon-based therapy, regimens that are more affordable and effective for HCV infections of all genotypes and difficult-to-treat patients are still challenging (D'Ambrosio et al., 2017; Li and De Clercq, 2017).

Proprotein convertase subtilisin/kexin type 9 (PCSK9), the ninth member in the proprotein convertase family, is primarily produced in the liver and intestine (Norata et al., 2014; Seidah et al., 2014). It is a 72 kDa protein that is composed of a signal peptide (SP), a prodomain (PD), a catalytic domain and a C-terminal domain (Du et al., 2011; Marais et al., 2012; Seidah et al., 2014). After removal of SP by signal peptidase from preproPCSK9, the proPCSK9 is translocated to the ER, where PD is auto-catalytically cleaved to produce mature PCSK9 (63 kDa). PD cleavage is essential for maturation and secretion of PCSK9, but cleaved PD is still associated with mature PCSK9 through hydrogen bonds, which forms a PCSK9-PD complex. After exiting the ER, the complex is routed to *cis*-Golgi and secreted outside of the cell (Farnier, 2014; Lambert et al., 2009; Poirier and Mayer, 2013; Seidah et al., 2013). PCSK9 can down-regulate low-density lipoprotein receptor (LDLR) by facilitating LDLR lysosomal degradation. Thus, it plays an essential role in increasing the plasma level of low-density lipoprotein

cholesterol (LDLC), which is a determinant for familial autosomal dominant hypercholesterolemia (Lambert et al., 2009; Tibolla et al., 2011).

Apart from the major role of PCSK9 in the regulation of LDLC homeostasis, it has been published that PCSK9 has an antiviral effect on HCV. Labonte et al. were the first to demonstrate that PCSK9 could inhibit HCV entry and subsequent replication (Labonte et al., 2009). They identified that PCSK9 markedly decreases the levels of HCV receptors LDLR and cluster of differentiation 81 at the cell surface. In a follow-up study, Syed et al. characterized the effect of PCSK9 on HCV replication specifically using an approach that could overcome the inhibition of HCV entry by PCSK9 (Syed et al., 2014). They showed that PCSK9 inhibits HCV replication in HCV replicon cells and after HCV infection (Syed et al., 2014). However, the underlying molecular mechanisms have not been studied. In addition, the effects of PCSK9 on HCV RNA translation, virion assembly and secretion have not been characterized. A thorough understanding of the modulation of the HCV life cycle by PCSK9 will provide important information not only for developing PCSK9 as an alternative and/or supplementary HCV therapy, but also for using PCSK9 inhibitors to treat cardiovascular diseases especially in hepatitis C patients.

In this paper, we further studied the role of PCSK9 in the HCV life cycle. We demonstrated that PCSK9 had no effect on HCV translation, virion assembly or secretion. PCSK9 down-regulated HCV replication with the involvement of PCSK9 auto-cleavage. Furthermore, we showed an interaction between PCSK9 and NS5A, and the interaction decreased NS5A dimerization and RNA binding activity of NS5A.

3.5 Material and Methods

3.5.1 Plasmids and *in vitro* transcription

Wild-type and replication-deficient pFLneo-J6/JFH-1(p7-RLuc-2A) plasmids containing the full-length HCV-2a J6/JFH-1 genomic sequence with an internal Renilla luciferase reporter gene were obtained from Dr. Charles Rice (Jones et al., 2007). Genomic plasmid pFLneo-J6 core^{Flag}/JFH-1(p7-RLuc-2A) and subgenomic replicon (SGR) plasmid RLuc-HCV-2a JFH-1 NS3-NS5B were described previously (Wu et al., 2017). A Flag-tag sequence was inserted in the NS5A coding region in the SGR backbone as previously described (Hazari et al., 2010), resulting in plasmid RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B. The coding sequence for the aa. 95-215 of NS5A was deleted to give rise to RLuc-HCV-2a JFH-1 NS3-Δaa. 95-215 NS5A^{Flag}-NS5B. A

replication-deficient SGR was generated by introduction of GNN mutation in NS5B, generating plasmid RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B GNN. To generate plasmids expressing HCV-2a JFH-1 NS5A or glutathione S-transferase (GST) with a Flag-tag, the respective coding sequences were amplified by PCR and cloned into the pEF vector. Plasmids expressing NS5A domains and different truncations with a Myc-tag were also generated. Plasmids expressing PCSK9 and its individual domains with a C-terminal V5-tag were received from Dr. Daping Fan (Du et al., 2011; Fan et al., 2008). Using these plasmids as templates, we generated plasmids expressing PCSK9 and ΔPD PCSK9 with a Flag-tag. Plasmids expressing Flag-tagged wild-type, D374Y or Δaa. 31-52 mutant PCSK9 were received from Dr. Thomas Lagace (Kosenko et al., 2013). PCSK9 Q152H mutant was generated by site-directed mutagenesis. To generate a plasmid expressing LDLR with a Myc-tag, the coding sequence of LDLR was amplified by PCR using a human LDLR cDNA (Transomic) as the template and cloned into the pEF vector. To acquire purified proteins from *E. coli*, the coding sequences for HCV-2a JFH-1 NS5A or PCSK9 were cloned into the pT7 His_6-SUMO vector. HCV-2a J6 core- and red fluorescent protein (RFP)-expressing plasmids in the pT7 His_6-SUMO vector were described previously (Wu et al., 2017). The pLKO.1 lentiviral plasmid expressing PCSK9-specific shRNA with the target sequence 5'-TACACACGTGTTGTCTACGGC-3' was purchased from Dharmacon/GE Healthcare. Non-silencing control or GFP siRNA-encoding pLKO.1 lentiviral plasmids were Addgene plasmids 10879 and 12273, respectively. To generate an shRNA-resistant PCSK9 cDNA without changing the amino acid sequence, the shRNA target sequence was mutated to TACACA<u>T</u>GT<u>A</u>TTGTC<u>G</u>ACGGC by site-directed mutagenesis. The mutated nucleotides were underlined. For split luciferase complementation assay (SLCA), plasmids encoding amino acids 1-229 or 230-311 of the Renilla luciferase protein, p-LN-C, p-C-LN, p-LC-C, and p-C-LC, were provided by Dr. Feng Li (Deng et al., 2011) and transferred to pcDNA3 vector. Coding sequences for Myc-tagged full-length NS5A or NS5A without amino acids 95-215 were cloned into the SLCA plasmids.

For *in vitro* transcription, plasmids encoding HCV-2a J6/JFH-1(p7-RLuc2A) GNN, RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B, RLuc-HCV-2a JFH-1 NS3-Δaa. 95-215 NS5A^{Flag}-NS5B, or RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B GNN were linearized by *Xba*I (New England Biolabs) and then *in vitro* transcribed by MEGAscript T7 Transcription Kit (Thermo Fisher Scientific).

3.5.2 Cell lines, transfection, luciferase assay and Western blotting

Huh-7, Huh-7.5, and human embryonic kidney HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (MilliporeSigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (MilliporeSigma) at 37 °C and 5% CO_2. Huh-7-HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon, Huh-7-HCV-2a J6 core^{Flag}/JFH-1(p7-RLuc-2A) reporter genomic replicon, and Huh-7-RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B SGR replicon cells were generated as previously described (Hundt et al., 2015; Wu et al., 2017). Cells were transfected with plasmid DNA and HCV reporter RNA using calcium phosphate or the jetPEI reagent (Polyplus). For the luciferase assay, cells were lysed in Passive Lysis Buffer (Promega) and luciferase activity was measured using Luciferase Assay System (Promega) by GloMax 20/20 Luminometer (Promega) according to the manufacturer's protocol. Luciferase activity was normalized to total protein concentrations which were determined by a Bradford protein assay (Bio-Rad). For Western blotting, cells were lysed in SDS sample buffer. Cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBS for 1 h at room temperature and then incubated with indicated primary antibodies overnight at 4 °C. After incubating with infrared dye-labeled secondary antibodies for 1 h at room temperature, the blots were scanned using Odyssey CLx Imaging System (Li-Cor Biosciences). These antibodies were used: Flag-tag (MilliporeSigma), Myc-tag, V5-tag and β-actin (Cell Signaling Technology), His_6-tag (Qiagen), NS5A (Huang et al., 2004), IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

3.5.3 Reverse transcription quantitative real-time PCR

Total RNA was extracted from cells using TRIzol (Thermo Fisher Scientific) and digested with DNase I (Thermo Fisher Scientific). Then RNA was reverse transcribed into cDNA by SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). The final step of reverse transcription quantitative real-time PCR (RT-qPCR) was carried out by iQ5 real-time PCR detection system (Bio-Rad) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with primers HCV-FD (5'-AGAGCCATAGTGGTCTGCGGAAC-3') and HCV-Rev (5'-CCTTTCGCAACCCAACGCTACTC-3'). HCV RNA levels were normalized with house-keeping gene β-glucuronidase (GUSB) RNA levels using primers GUSB-FD (5'-

GGTGCTGAGGATTGGCAGTG-3') and GUSB-Rev (5'-CGCACTTCCAACTTGAACAGG-3') by $2^{-\Delta\Delta Ct}$ method (Wu et al., 2017).

3.5.4 HCV infection, virion assembly and secretion assay

For HCV infection, cell-culture derived HCV (HCVcc) was collected from the supernatant of Huh-7-HCV-2a J6 $core^{Flag}$/JFH-1(p7-RLuc-2A) reporter genomic replicon cells and concentrated using Amicon Ultra-15 Centrifugal Filter Units (MilliporeSigma). Virus titer was determined as per an established protocol (Yi, 2010). Naïve Huh-7.5 cells were infected with HCVcc at a multiplicity of infection (MOI) of 0.05 for 4 h and fresh media was replaced. To examine the effect of PCSK9 on HCV virion assembly, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, GST- or PCSK9-expressing plasmids. After 48 h, cells were washed and harvested in fresh media, followed by three cycles of freezing and thawing. Cell debris was removed and the supernatants were used to infect naïve Huh-7.5 cells for 4 h. Luciferase assay was performed 72 h after infection. To examine the effect of PCSK9 on HCV virion secretion, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, GST- or PCSK9-expressing plasmids. After 48 h, the supernatant was collected and concentrated using Amicon Ultra-15 Centrifugal Filter Units. Naïve Huh-7.5 cells were infected for 4 h and luciferase assay performed 72 h after infection. Luciferase activity was normalized to HCV RNA levels of vector-, GST- or PCSK9-transfected HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells and total protein concentrations.

3.5.5 Co-immunoprecipitation, RNA and protein immunoprecipitation assays

For co-immunoprecipitation (co-IP) assay, cells were lysed in a lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche) at 4 ºC. Cell lysates were immunoprecipitated with indicated antibodies using Dynabeads Protein G (Thermo Fisher Scientific) according to the manufacturer's protocol. Immunoprecipitated proteins were eluted in 2×SDS sample buffer and then subjected to SDS-PAGE and Western blotting. For RNA and protein immunoprecipitation (RIP) assay, cells were lysed in a lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) supplemented with protease inhibitor cocktail, 50 µg/mL BSA, 10 µg/mL yeast tRNA (Thermo Fisher Scientific) and 200 U/mL RNaseOUT (Thermo Fisher

Scientific) at 4 ºC. Cell lysates were immunoprecipitated with indicated antibodies using Dynabeads Protein G. Immunoprecipitated RNA was extracted using TRIzol and then subjected to RT-qPCR.

3.5.6 Protein expression and purification

E. coli BL21 (DE3) strain was transformed with His_6-tagged HCV core, NS5A, PCSK9 or RFP expressing plasmids and cultured in LB media at 37ºC until OD_{595nm} reached 0.5. Then 0.1 mM IPTG (Thermo Fisher Scientific) was added and the culture was incubated overnight at 21 ºC. The expressed proteins were purified by nickel-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer's protocol. The purified proteins were buffer exchanged to PBS and concentrated using Amicon Ultra-15 Centrifugal Filter Units.

3.5.7 Immunofluorescence assay and confocal microscopy

Cells in eight-well chamber slides were fixed with 4% paraformaldehyde for 20 min. After permeabilized with 0.1% Triton X-100 in PBS for 10 min, cells were blocked with 1% goat serum (Thermo Fisher Scientific) in PBS for 30 min. Then cells were incubated with indicated primary antibodies for 2 h, followed by Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h. Next, cells were stained with 300 nM DAPI (Thermo Fisher Scientific) for 5 min and 0.3 mg/mL Oil Red O (Alfa Aesar) for 10 min as previously described (Jackel-Cram et al., 2007). After washing and air-drying, cells were mounted with ProLong antifade reagent (Thermo Fisher Scientific). All steps were performed at room temperature. Cells were visualized and pictures were taken using a Leica TCS SP5 confocal microscope (Leica Microsystems). Pearson's correlation coefficients that represent the overlap of two proteins or protein and lipid droplets were calculated using the Fiji software.

3.5.8 Statistical analysis

All experiments were performed in triplicate and data analyzed by GraphPad Prism 7. Statistical differences were determined by Student's *t*-test, one- or two-way analysis of variance (ANOVA). Statistical significance was demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, *** if $p < 0.001$, **** if $p < 0.0001$, or *NS* for not significant.

3.6 Results

3.6.1 PCSK9 has no effect on HCV translation, virion assembly or secretion

To study the effect of PCSK9 on HCV translation, Huh-7 cells were co-transfected with HCV-2a J6/JFH-1(p7-RLuc2A) GNN reporter RNA and vector, or plasmids expressing PCSK9 or GST (as a negative control). The conserved GDD motif in NS5B polymerase is mutated to GNN, which renders this HCV RNA replication deficient (Jones et al., 2007). Thus, Renilla luciferase activity reflects HCV RNA translation level (Hoffman et al., 2015). As shown in Figure 3.1A, the luciferase activity measured after expression of PCSK9 was similar to those after vector transfection or GST expression. The levels of NS5A protein determined by Western blotting were also similar in vector-, GST- and PCSK9-transfected cells (Figure 3.S1), confirming the luciferase reporter data. These results suggested that PCSK9 did not affect HCV translation.

To study the effect of PCSK9 on HCV virion assembly, Huh-7-HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, GST- or PCSK9-expressing plasmids. Cells were washed to remove secreted HCV virions, and lysed in fresh media. After three cycles of freezing and thawing, cell debris was removed and the supernatants were used to infect naïve Huh-7.5 cells. Then luciferase assay was performed to determine the amounts of virions assembled in the initial genomic replicon cells. As shown in Figure 3.1B, PCSK9 expression did not cause significant change in luciferase activity compared to vector or GST expression, indicating that PCSK9 did not affect virion assembly. To determine the effect of PCSK9 on HCV virion secretion, we used the supernatant of HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells which were transfected with vector, GST- or PCSK9-expressing plasmids to infect naïve Huh-7.5 cells. Similarly, luciferase assay results showed that PCSK9 did not alter virion secretion (Figure 3.1C). The expression of GST and PCSK9 proteins was confirmed by Western blotting (Figure 3.1D).

Taken together, these results indicated that PCSK9 did not affect HCV translation, virion assembly or secretion.

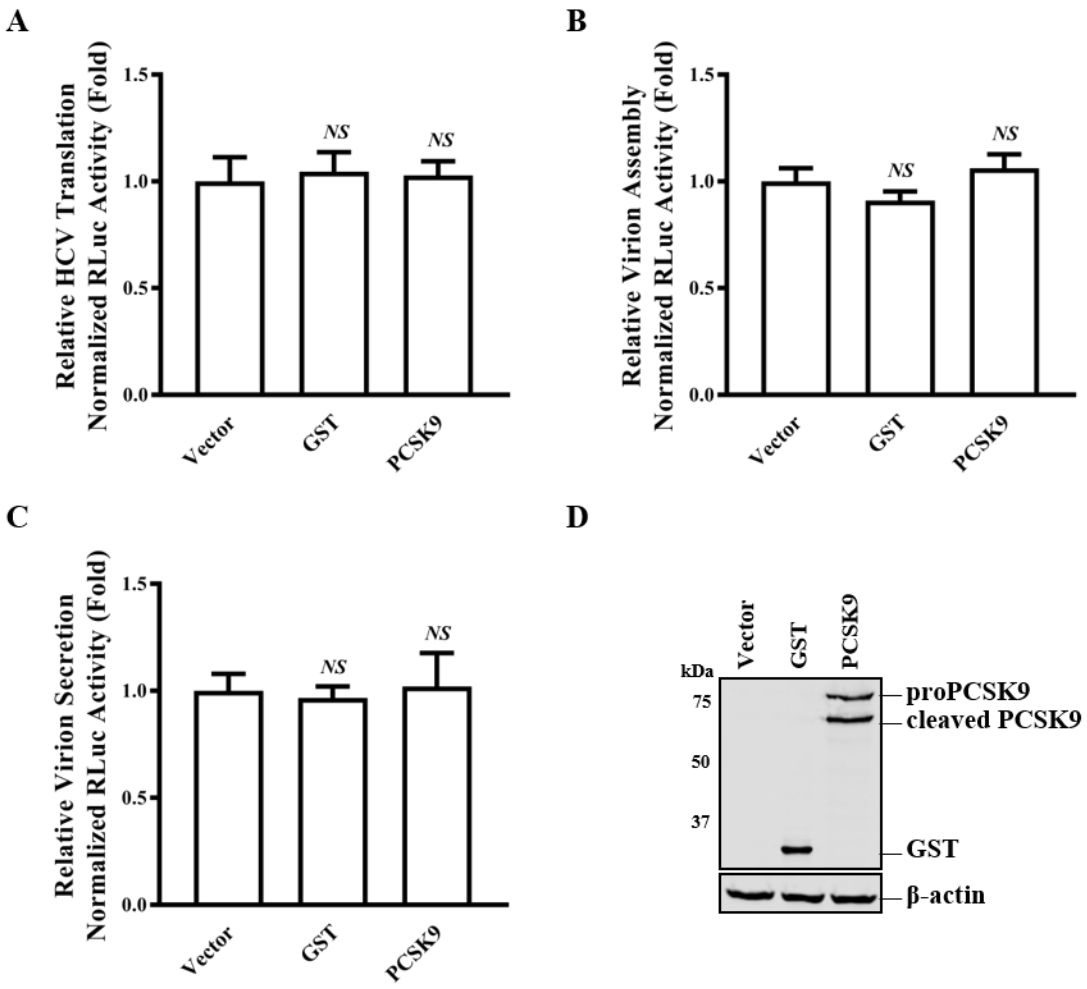


Figure 3.1 PCSK9 does not affect HCV translation, virion assembly or secretion. A, Huh-7 cells were co-transfected with HCV-2a J6/JFH-1(p7-RLuc2A) GNN reporter RNA and vector, or plasmids expressing Flag-tagged GST or PCSK9. Luciferase assay was performed 24 h after transfection. **B**, Huh-7-HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, or plasmids expressing Flag-tagged GST or PCSK9. After 48 h, cells were washed and harvested in fresh media. After three cycles of freezing and thawing, cell debris was removed and the supernatants were used to infect naïve Huh-7.5 cells. Luciferase assay was performed 72 h after infection. **C**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, or plasmids expressing Flag-tagged GST or PCSK9. After 48 h, the supernatants were collected, concentrated, and used to infect naïve Huh-7.5 cells. Luciferase assay was performed 72 h after infection. *NS*, not significant. **D**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, or plasmids expressing Flag-tagged GST or PCSK9. Cell lysates collected 48 h after transfection were subjected to Western blotting with Flag and β-actin antibodies.

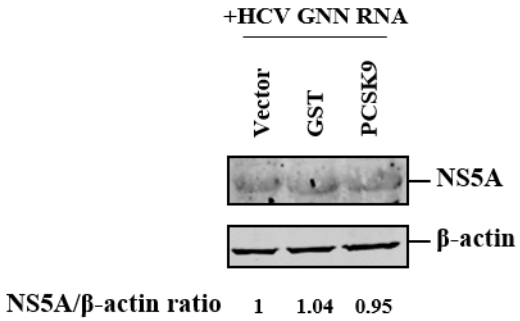


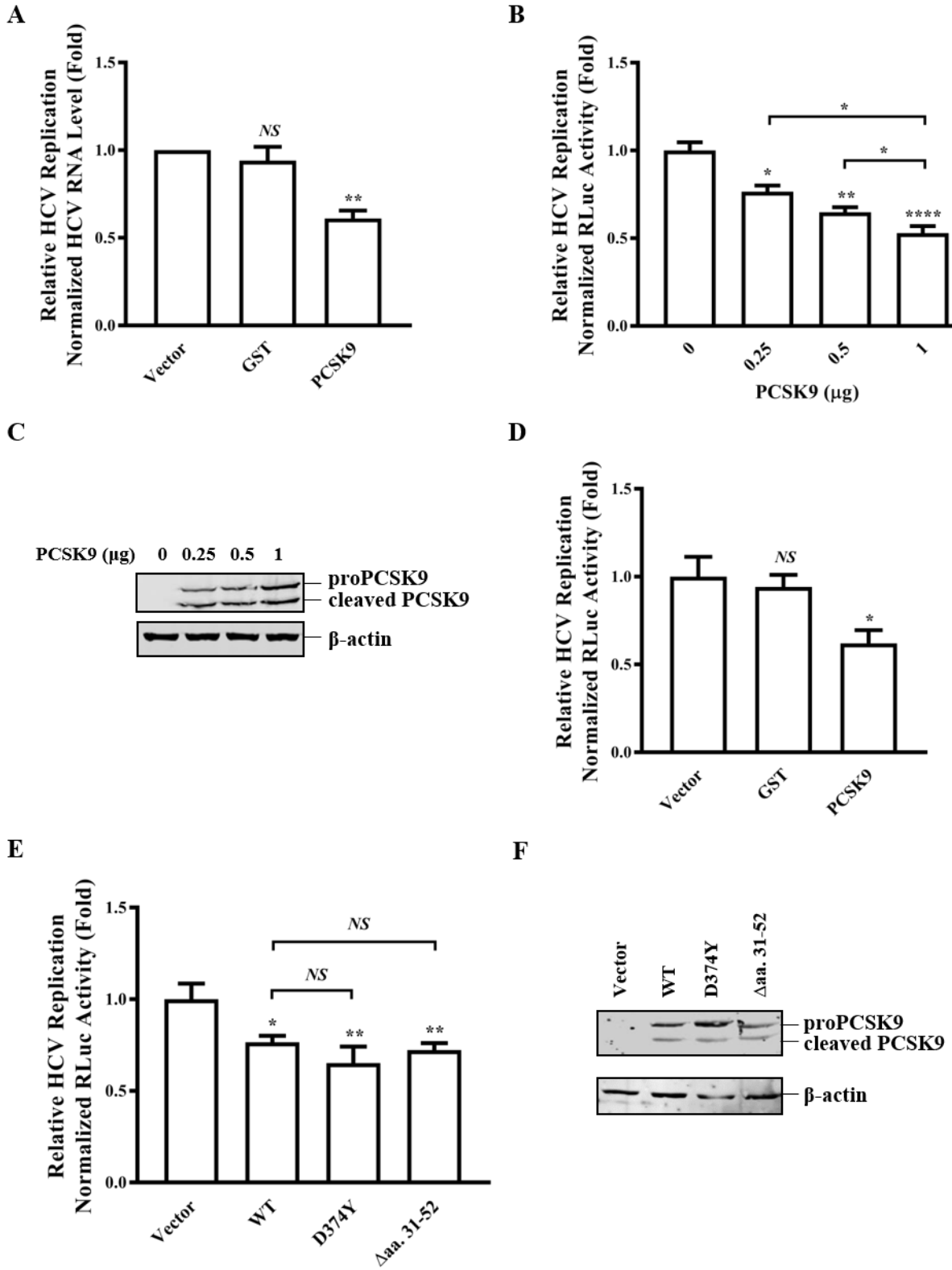
Figure 3.S1 PCSK9 does not affect HCV translation. Huh-7 cells were co-transfected with HCV-2a J6/JFH-1(p7-RLuc2A) GNN reporter RNA and vector, or plasmids expressing Flag-tagged GST or PCSK9. Cell lysates collected 24 h after transfection were subjected to Western blotting with NS5A and β-actin antibodies. NS5A expression was indicated by normalizing band intensities of NS5A to β-actin.

3.6.2 PCSK9 overexpression inhibits HCV replication in a dose-dependent manner

Although PCSK9 has been shown to inhibit HCV replication (Syed et al., 2014), the mechanism has not been well characterized. With a focus on the mechanisms, we studied the effect of PCSK9 on HCV replication. To this end, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with plasmid vector, GST- or PCSK9-expressing plasmids, and HCV RNA levels were measured by RT-qPCR. As shown in Figure 3.2A, the HCV RNA level in PCSK9-transfected cells significantly reduced compared to vector- or GST-transfected cells, suggesting that PCSK9 down-regulated HCV replication. Consistently, the NS5A levels also decreased in PCSK9-transfected cells (Figure 3.S2A). Since PCSK9 does not affect HCV translation, HCV protein level should reflect the degree of HCV replication. To demonstrate whether the levels of PCSK9 protein were inversely correlated with HCV replication, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with increasing amounts of PCSK9-expressing plasmid. Results in Figure 3.2B showed a dose-dependent decrease in Renilla luciferase activity with increasing levels of PCSK9 as shown in Figure 3.2C. Correspondingly, the levels of NS5A gradually reduced as PCSK9 gradually increased (Figure 3.S2B). These results indicated that PCSK9 inhibited HCV replication in a dose-dependent manner. Next, we studied the effect of PCSK9 on HCV replication after HCV infection. Huh-7.5 cells were infected with HCVcc before being transfected with plasmid vector, GST- or PCSK9-expressing plasmids, which eliminated the effect of PCSK9 on HCV entry. The luciferase activity in PCSK9-transfected cells showed significant down-regulation compared to vector- or GST-transfected cells, indicating that PCSK9 inhibited HCV replication after HCV infection (Figure 3.2D).

3.6.3 PCSK9-induced LDLR degradation does not affect HCV replication

As published in other work, LDLR plays an important role in HCV replication because an LDLR-specific antibody that blocks LDLR suppresses HCV replication (Albecka et al., 2012). Since one of the major functions of PCSK9 is to down-regulate LDLR, we investigated whether LDLR degradation by PCSK9 was involved in HCV replication inhibition by PCSK9. We took advantage of two PCSK9 mutants that have been characterized as either gain-of-function (D374Y) or loss-of-function (Δaa. 31-52) mutants in terms of LDLR degradation. D374Y PCSK9


A
Relative HCV Replication
Normalized HCV RNA Level (Fold)
1.5
1.0
0.5
0.0
NS
**
Vector
GST
PCSK9
B
Relative HCV Replication
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
*
*
*
**
****
0
0.25
0.5
1
PCSK9 (μg)
C
PCSK9 (μg) 0 0.25 0.5 1
proPCSK9
cleaved PCSK9
β-actin
D
Relative HCV Replication
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
NS
*
Vector
GST
PCSK9
E
Relative HCV Replication
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
NS
NS
*
**
**
Vector
WT
D374Y
Δaa. 31-52
F
Vector
WT
D374Y
Δaa. 31-52
proPCSK9
cleaved PCSK9
β-actin

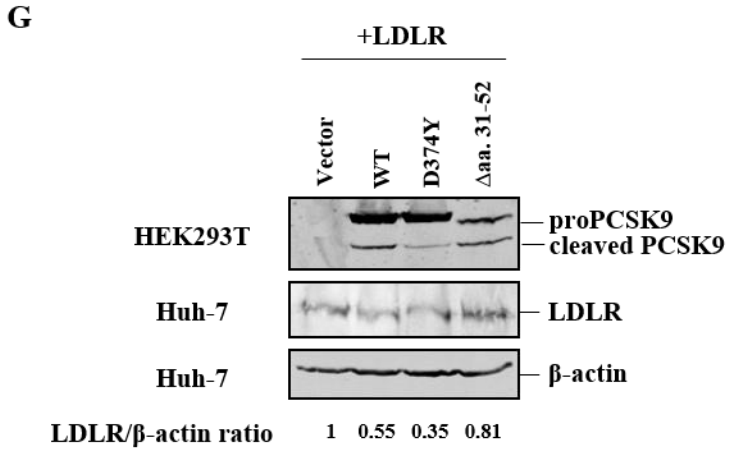


Figure 3.2 Inhibition of HCV replication by PCSK9 is dose-dependent and LDLR-independent. **A**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, or plasmids expressing Flag-tagged GST or PCSK9. After 48 h, RNA was isolated and RT-qPCR was performed using HCV-specific primers. Relative HCV RNA levels were shown after normalization against those of GUSB. **B** and **C**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector or increasing amounts of Flag-PCSK9-expressing plasmid. Luciferase assay was performed 48 h after transfection (**B**). Cell lysates collected 48 h after transfection were subjected to Western blotting with Flag and β-actin antibodies (**C**). **D**, Huh-7.5 cells were infected with HCVcc at an MOI of 0.05. At 24 h post infection, cells were transfected with vector, or plasmids expressing Flag-tagged GST or PCSK9. Luciferase assay was performed 48 h after transfection. **E** and **F**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, or plasmids expressing Flag-tagged wild-type, D374Y or Δaa. 31-52 PCSK9. Luciferase assay was performed 48 h after transfection (**E**). Cell lysates collected 48 h after transfection were subjected to Western blotting with Flag and β-actin antibodies (**F**). **G**, HEK293T cells were transfected with vector, or plasmids expressing Flag-tagged wild-type, D374Y or Δaa. 31-52 PCSK9. The supernatants collected 48 h after transfection were added to Myc-LDLR-transfected Huh-7 cells and incubated overnight. Both HEK293T and Huh-7 cell lysates were used in Western blotting with Flag, Myc and β-actin antibodies. LDLR expression was indicated by normalizing band intensities of LDLR to β-actin. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, **** if $p < 0.0001$, or *NS* for not significant.

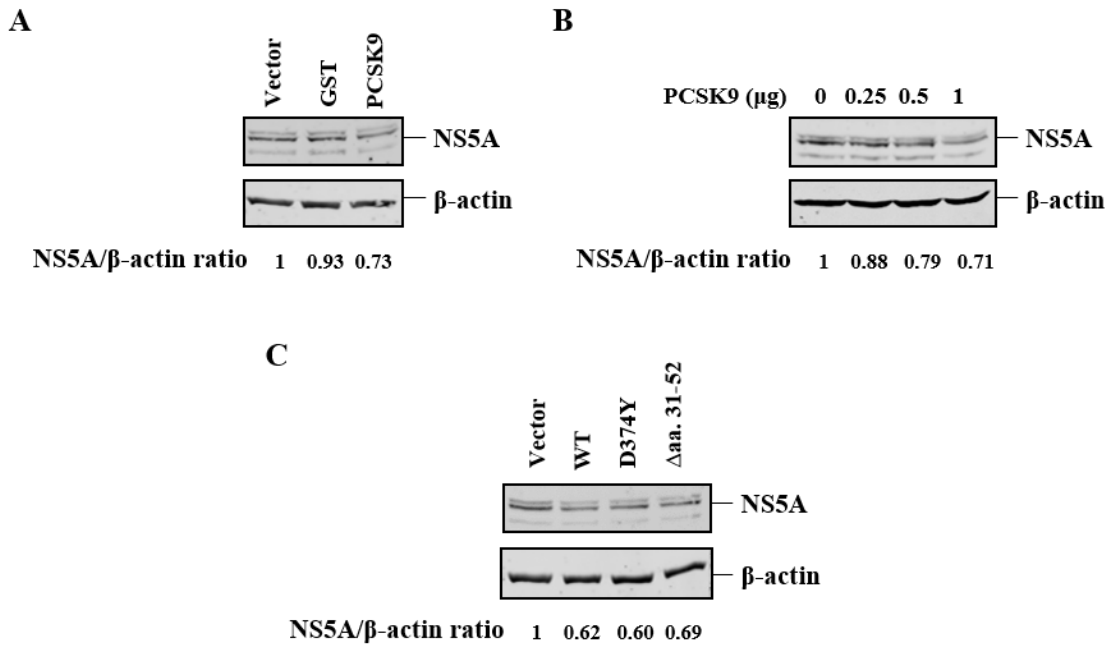


Figure 3.S2 Inhibition of HCV replication by PCSK9 is dose-dependent and LDLR-independent. **A**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, or plasmids expressing Flag-tagged GST or PCSK9. **B**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector or increasing amounts of Flag-PCSK9-expressing plasmid. **C**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, or plasmids expressing Flag-tagged wild-type, D374Y or Δaa. 31-52 PCSK9. Cell lysates collected 48 h after transfection were subjected to Western blotting with NS5A and β-actin antibodies. NS5A expression was indicated by normalizing band intensities of NS5A to β-actin.

increases LDLR degradation, while Δaa. 31-52 PCSK9 decreases LDLR degradation. Both mutants still retain the capacity for auto-cleavage and secretion (Du et al., 2011; Dwyre et al., 2011; Kosenko et al., 2013). We transfected HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells with plasmids expressing PCSK9, wild-type, D374Y, or Δaa. 31-52, and measured HCV replication. The results showed that D374Y PCSK9 and Δaa. 31-52 PCSK9 expression had similar inhibitory effects on HCV replication in comparison to wild-type PCSK9 (Figure 3.2E for RLuc activities and 3.S2C for NS5A levels). The expression of wild-type and mutant PCSK9 proteins was demonstrated by Western blotting (Figure 3.2F). To confirm the effects of these two PCSK9 mutants on LDLR degradation, we transfected HEK293T cells with these PCSK9 plasmids and collected the supernatants from transfected HEK293T cells to treat Myc-LDLR-transfected Huh-7 cells as per an established protocol (Poirier et al., 2016). Figure 3.2G showed that, in comparison to wild-type PCSK9, D374Y PCSK9 increased, while Δaa. 31-52 PCSK9 decreased LDLR degradation. These results suggested that HCV replication inhibition by PCSK9 was not due to LDLR degradation.

3.6.4 PCSK9 knockdown increases HCV replication

To further confirm the role of PCSK9 in HCV replication, we knocked down endogenous PCSK9 in HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells by PCSK9-specific shRNA and measured HCV replication. As shown in Figure 3.3A and 3.S3A, knocking down PCSK9 resulted in significantly higher luciferase activity and NS5A level in comparison to control or GFP knockdown. The level of PCSK9 protein after shRNA knockdown was demonstrated by Western blotting (Figure 3.3B). To confirm that the observed changes in HCV replication after PCSK9 knockdown were indeed due to the altered PCSK9 levels, we generated a PCSK9-expressing plasmid with the shRNA target sequence mutated (ΔshRNA PCSK9). We co-transfected HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells with control or PCSK9 shRNA, together with plasmids expressing wild-type or ΔshRNA PCSK9. As expected, when co-transfecting control or PCSK9 shRNA with wild-type PCSK9, HCV RNA replication represented by luciferase activity and NS5A level in PCSK9 knockdown cells increased compared to control cells (Figure 3.3C and 3.S3B). When overexpressing ΔshRNA PCSK9 that could not be knocked down by the shRNA, PCSK9 shRNA no longer up-regulated HCV replication compared to control shRNA (Figure 3.3C and 3.S3B). PCSK9 levels in these cells

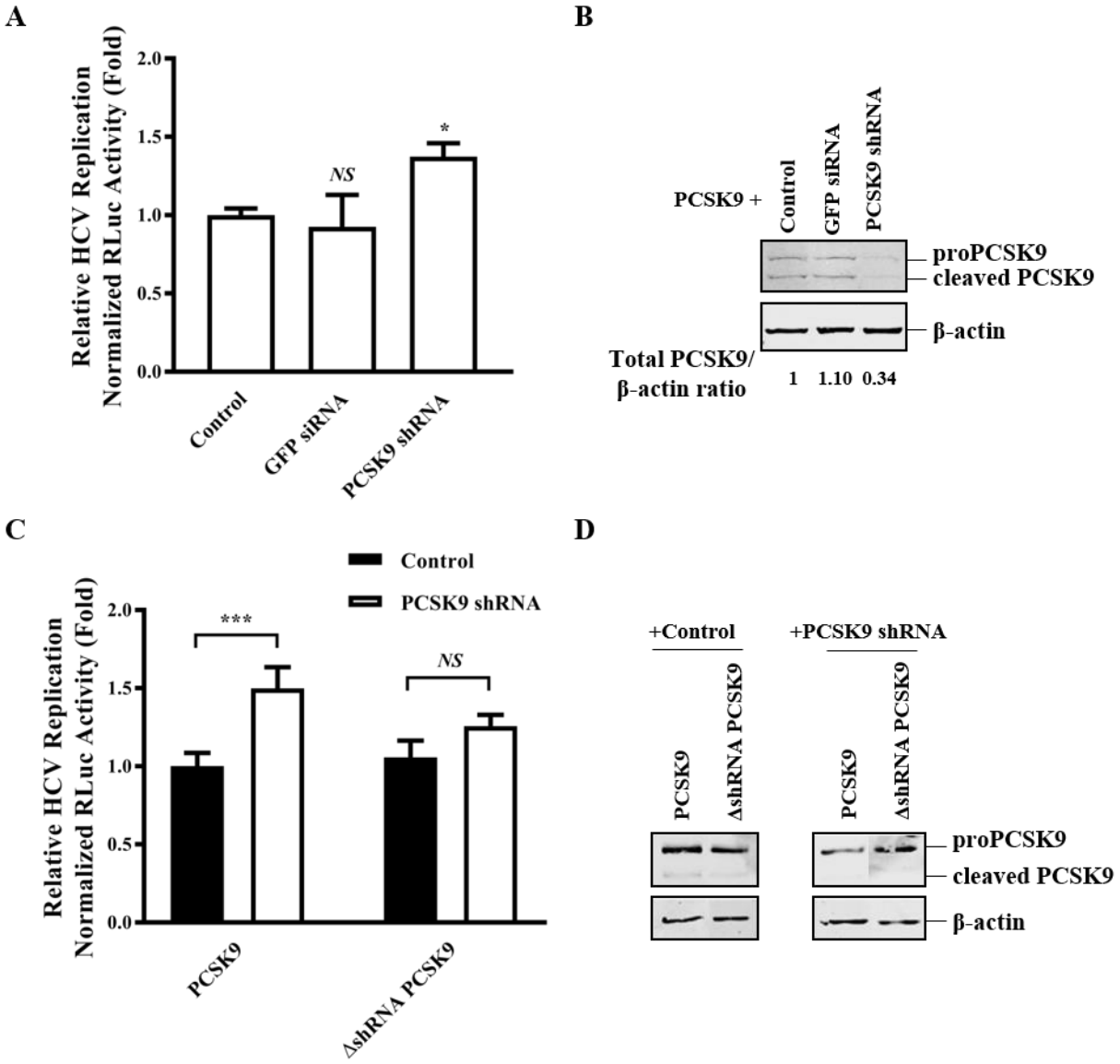

A
Relative HCV Replication
Normalized RLuc Activity (Fold)
2.0
1.5
1.0
0.5
0.0
NS
*
Control
GFP siRNA
PCSK9 shRNA
B
PCSK9 +
Control
GFP siRNA
PCSK9 shRNA
proPCSK9
cleaved PCSK9
β-actin
Total PCSK9/
β-actin ratio
1
1.10
0.34
C
Control
PCSK9 shRNA
Relative HCV Replication
Normalized RLuc Activity (Fold)
2.0
1.5
1.0
0.5
0.0
***
NS
PCSK9
ΔshRNA PCSK9
D
+Control
+PCSK9 shRNA
PCSK9
ΔshRNA PCSK9
PCSK9
ΔshRNA PCSK9
proPCSK9
cleaved PCSK9
β-actin

Figure 3.3 PCSK9 knockdown up-regulates HCV replication. A, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with control, or plasmids expressing GFP siRNA or PCSK9 shRNA. Luciferase assay was performed 24 h after transfection. **B**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were co-transfected with plasmids expressing Flag-tagged PCSK9 and control, GFP siRNA or PCSK9 shRNA. Cell lysates collected 24 h after transfection were subjected to Western blotting with Flag and β-actin antibodies. PCSK9 expression was indicated by normalizing band intensities of total PCSK9 to β-actin. **C**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were co-transfected with plasmids expressing Flag-tagged wild-type or ΔshRNA PCSK9, together with control or PCSK9 shRNA. Luciferase assay was performed 48 h after transfection. **D**, Cell lysates collected 48 h after transfection in (C) were subjected to Western blotting with Flag and β-actin antibodies. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, *** if $p < 0.001$, or *NS* for not significant.

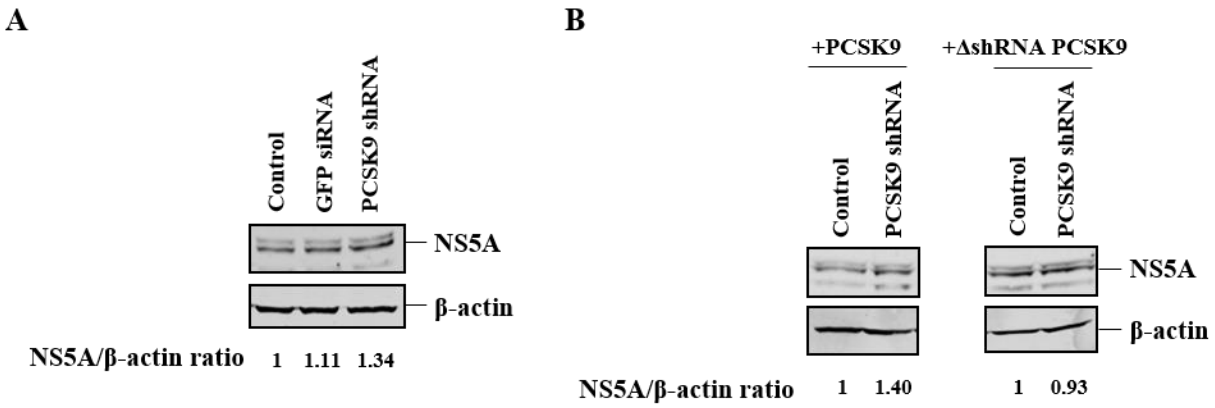


Figure 3.S3 PCSK9 knockdown up-regulates HCV replication. A, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with control, or plasmids expressing GFP siRNA or PCSK9 shRNA. Cell lysates collected 24 h after transfection were subjected to Western blotting with NS5A and β-actin antibodies. **B**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were co-transfected with plasmids expressing Flag-tagged wild-type or ΔshRNA PCSK9, together with control or PCSK9 shRNA. Cell lysates collected 48 h after transfection were subjected to Western blotting with NS5A and β-actin antibodies. NS5A expression was indicated by normalizing band intensities of NS5A to β-actin.

were demonstrated in Figure 3.3D. These results demonstrated that HCV RNA replication was indeed negatively regulated by PCSK9.

3.6.5 ProPCSK9 down-regulates HCV replication

Two forms of PCSK9 are present in cells: proPCSK9 and cleaved PCSK9 (Schulz et al., 2015). Since these two forms were consistently detected in our experiments (Figure 1-3), we were interested in determining which form contributed to HCV replication inhibition. To this end, we generated plasmids expressing either proPCSK9 (Q152H PCSK9) or cleaved PCSK9 by deleting the PD domain (ΔPD PCSK9). The Q152H mutant has an amino acid substitution at the cleavage site, which suppresses PCSK9 auto-cleavage and secretion (Mayne et al., 2011). HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with these plasmids. Plasmid vector or wild-type PCSK9-expressing plasmid were included as controls. Luciferase assay and Western blotting results showed that Q152H PCSK9 down-regulated HCV replication, but ΔPD PCSK9 did not (Figure 3.4A and 3.S4A). Figure 3.4B confirmed that Q152H PCSK9 could not be auto-cleaved and only proPCSK9 was detected, while ΔPD PCSK9 expressed only the cleaved form. These results indicated that only proPCSK9 functioned in inhibiting HCV replication.

To further confirm that auto-cleavage of PCSK9 played a role in regulating HCV replication, we performed a competition experiment by co-transfecting wild-type PCSK9 with increasing amounts of ΔPD PCSK9 into HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells. Figure 3.4D showed that proPCSK9 gradually increased with the increasing levels of ΔPD PCSK9, suggesting that cleaved PCSK9 inhibited the auto-cleavage of proPCSK9. Correspondingly, the down-regulation of HCV replication gradually enhanced with the increasing levels of proPCSK9 (Figure 3.4C and 3.S4B). It implied that the inhibition of PCSK9 auto-cleavage down-regulated HCV replication. To rule out the possibility that increasing cleaved PCSK9 levels affected HCV replication, luciferase activity was determined in HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells after transfecting with increasing amounts of ΔPD PCSK9-expressing plasmid. No difference in luciferase activity was detected (Figure 3.4E). Similarly, levels of NS5A protein were not affected (Figure 3.S4C). The expression of ΔPD PCSK9 was shown in Figure 3.4F.

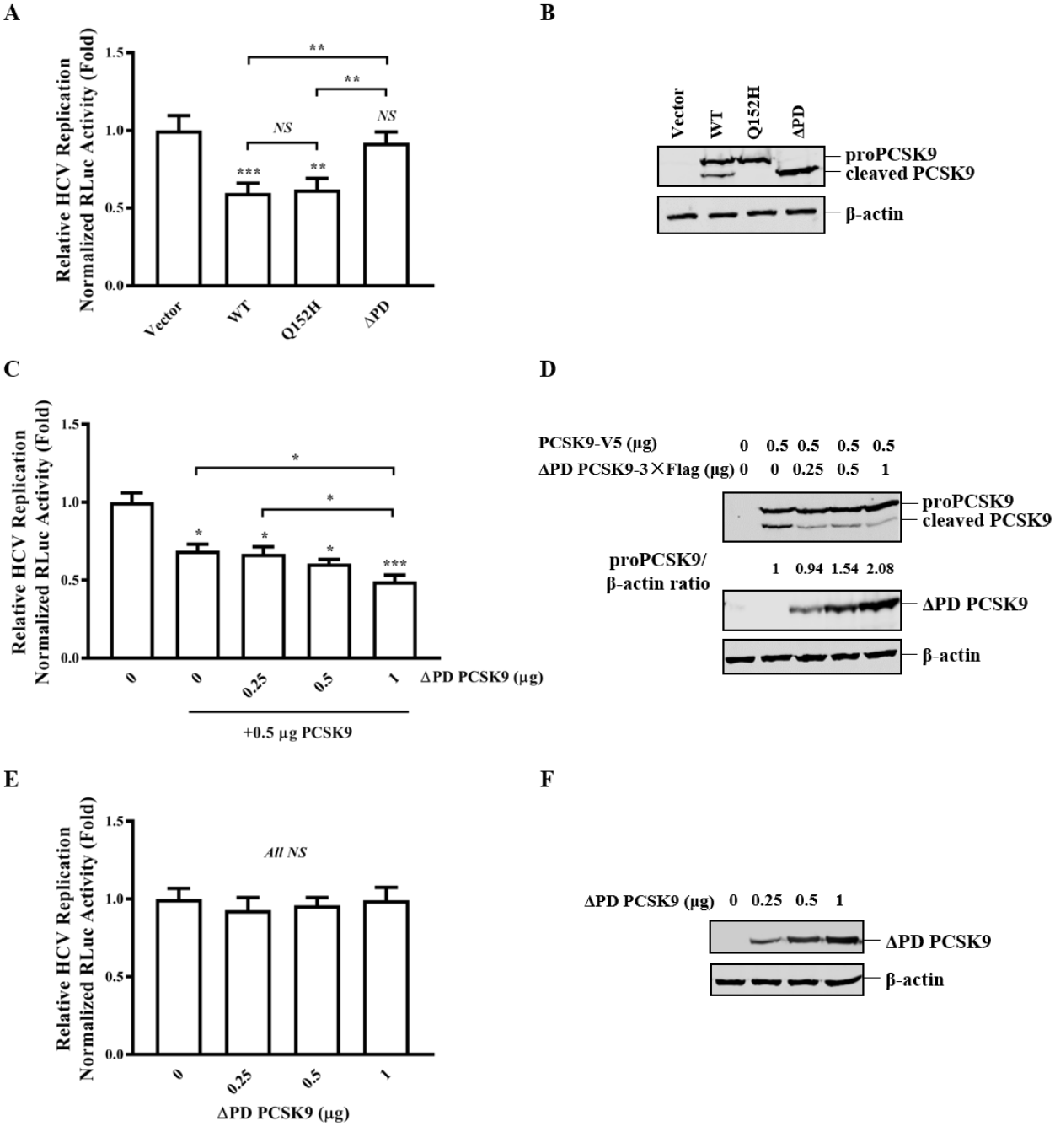

A
Relative HCV Replication
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
**
**
NS
NS
***
**
Vector
WT
Q152H
ΔPD
B
Vector
WT
Q152H
ΔPD
proPCSK9
cleaved PCSK9
β-actin
C
Relative HCV Replication
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
*
*
*
*
*
***
0
0
0.25
0.5
1
ΔPD PCSK9 (μg)
+0.5 μg PCSK9
D
PCSK9-V5 (μg) 0 0.5 0.5 0.5 0.5
ΔPD PCSK9-3×Flag (μg) 0 0 0.25 0.5 1
proPCSK9
cleaved PCSK9
proPCSK9/
β-actin ratio
1 0.94 1.54 2.08
ΔPD PCSK9
β-actin
E
Relative HCV Replication
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
All NS
0
0.25
0.5
1
ΔPD PCSK9 (μg)
F
ΔPD PCSK9 (μg) 0 0.25 0.5 1
ΔPD PCSK9
β-actin

Figure 3.4 ProPCSK9 down-regulates HCV replication. A, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, plasmids expressing Flag-tagged wild-type, Q152H or ΔPD PCSK9. Luciferase assay was performed 48 h after transfection. **B**, Cell lysates collected 48 h after transfection in (A) were subjected to Western blotting with Flag and β-actin antibodies. **C**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were co-transfected with plasmid expressing V5-tagged wild-type PCSK9 and increasing amounts of Flag-tagged ΔPD PCSK9-expressing plasmid. Luciferase assay was performed 48 h after transfection. **D**, Cell lysates collected 48 h after transfection in (C) were subjected to Western blotting with Flag, V5 and β-actin antibodies. ProPCSK9 expression was indicated by normalizing band intensities of proPCSK9 to β-actin. **E**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector or increasing amounts of Flag-tagged ΔPD PCSK9-expressing plasmid. Luciferase assay was performed 48 h after transfection. **F**, Cell lysates collected 48 h after transfection in (E) were subjected to Western blotting with Flag and β-actin antibodies. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, *** if $p < 0.001$, or *NS* for not significant.

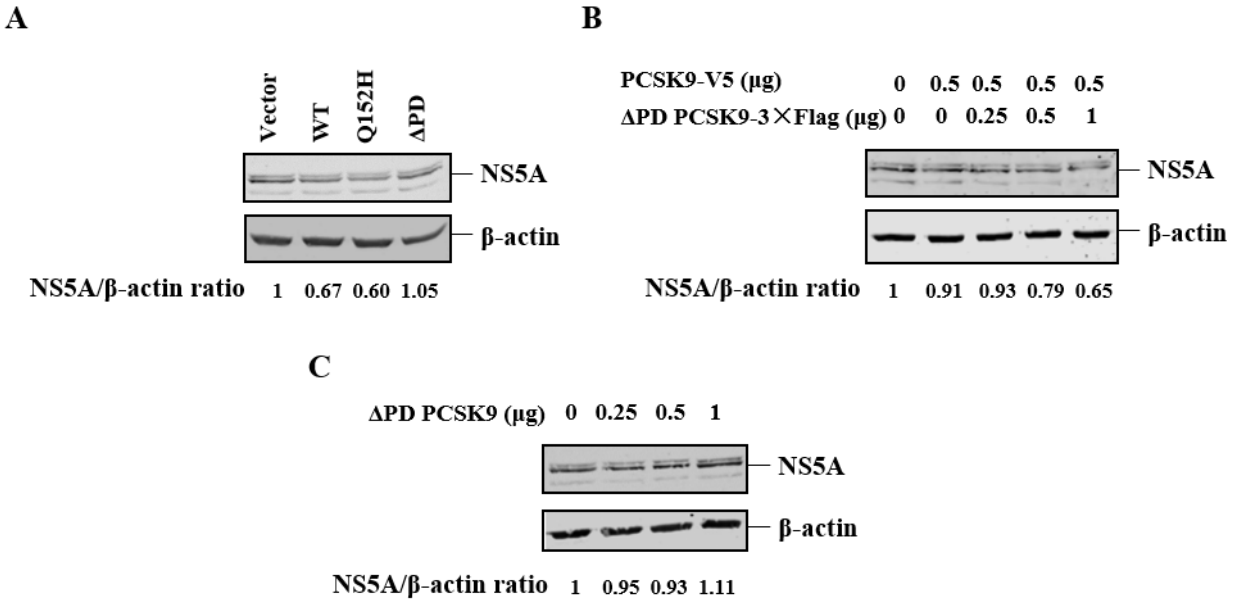


Figure 3.S4 ProPCSK9 down-regulates HCV replication. A, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector, plasmids expressing Flag-tagged wild-type, Q152H or ΔPD PCSK9. **B**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were co-transfected with plasmid expressing V5-tagged wild-type PCSK9 and increasing amounts of Flag-tagged ΔPD PCSK9-expressing plasmid. **C**, HCV-2a J6/JFH-1(p7-RLuc2A) reporter genomic replicon cells were transfected with vector or increasing amounts of Flag-tagged ΔPD PCSK9-expressing plasmid. Cell lysates collected 48 h after transfection were subjected to Western blotting with NS5A and β-actin antibodies. NS5A expression was indicated by normalizing band intensities of NS5A to β-actin.

Taken together, these results indicated that the inhibitory effect of PCSK9 on HCV replication might be negatively regulated by its auto-cleavage.

3.6.6 PCSK9 interacts and co-localizes with HCV NS5A

Next, we were interested in investigating how PCSK9 suppressed HCV replication. We reasoned that interaction between PCSK9 and HCV proteins might be a mechanism for PCSK9 to regulate HCV replication. NS5A is a phosphorylated zinc-metalloprotein required for RNA replication (Ross-Thriepland and Harris, 2015). Approximately 130 cellular proteins have been identified to interact with NS5A and the interactions often affect HCV replication (Cordek et al., 2011; Park et al., 2015; Ross-Thriepland and Harris, 2015). We therefore examined whether there was an interaction between PCSK9 and NS5A, thereby modulating HCV replication. For this purpose, we co-transfected HEK293T cells with Flag-tagged PCSK9 and Myc-tagged NS5A before the co-IP assay was performed. Plasmid vector and GST-expressing plasmid were used as negative controls. As shown in Figure 3.5A and B, while no GST was present in the immuoprecipitates, PCSK9 and NS5A were co-immunoprecipitated, suggesting an association between PCSK9 and NS5A. Figure 3.S5 confirmed that Flag-tagged PCSK9 or Myc-tagged NS5A could not be pulled down by the Protein G Dynabeads used in immunoprecipitation. We then determined whether PCSK9 and NS5A directly interacted with each other using purified PCSK9 and NS5A proteins in the co-IP assay. Purified HCV core and RFP were used as positive and negative controls for interacting with NS5A, respectively, because an interaction between core and NS5A has been reported (Gawlik et al., 2014; Lai et al., 2014). The results showed that NS5A could interact with PCSK9 or core, but not RFP, indicating a direct interaction between PCSK9 and NS5A (Figure 3.5C). Next, we examined the interaction in Huh-7-RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B SGR cells by transfecting with plasmids expressing V5-tagged PCSK9 or GST. Figure 3.5D showed that PCSK9, but not GST, could interact with NS5A in HCV-replicating cells. We noticed that NS5A interacted with proPCSK9, but not the cleaved PCSK9 (Figure 3.5B and D). To further confirm this observation, HEK293T cells were co-transfected with plasmids expressing Myc-tagged NS5A and Flag-tagged wild-type, or ΔPD PCSK9 (cleaved PCSK9). Interestingly, although there was an interaction between NS5A and ΔPD PCSK9, it appeared that NS5A only bound to the proPCSK9 form when both proPCSK9 and cleaved PCSK9 were present after expressing wild-type PCSK9 because only the proPCSK9

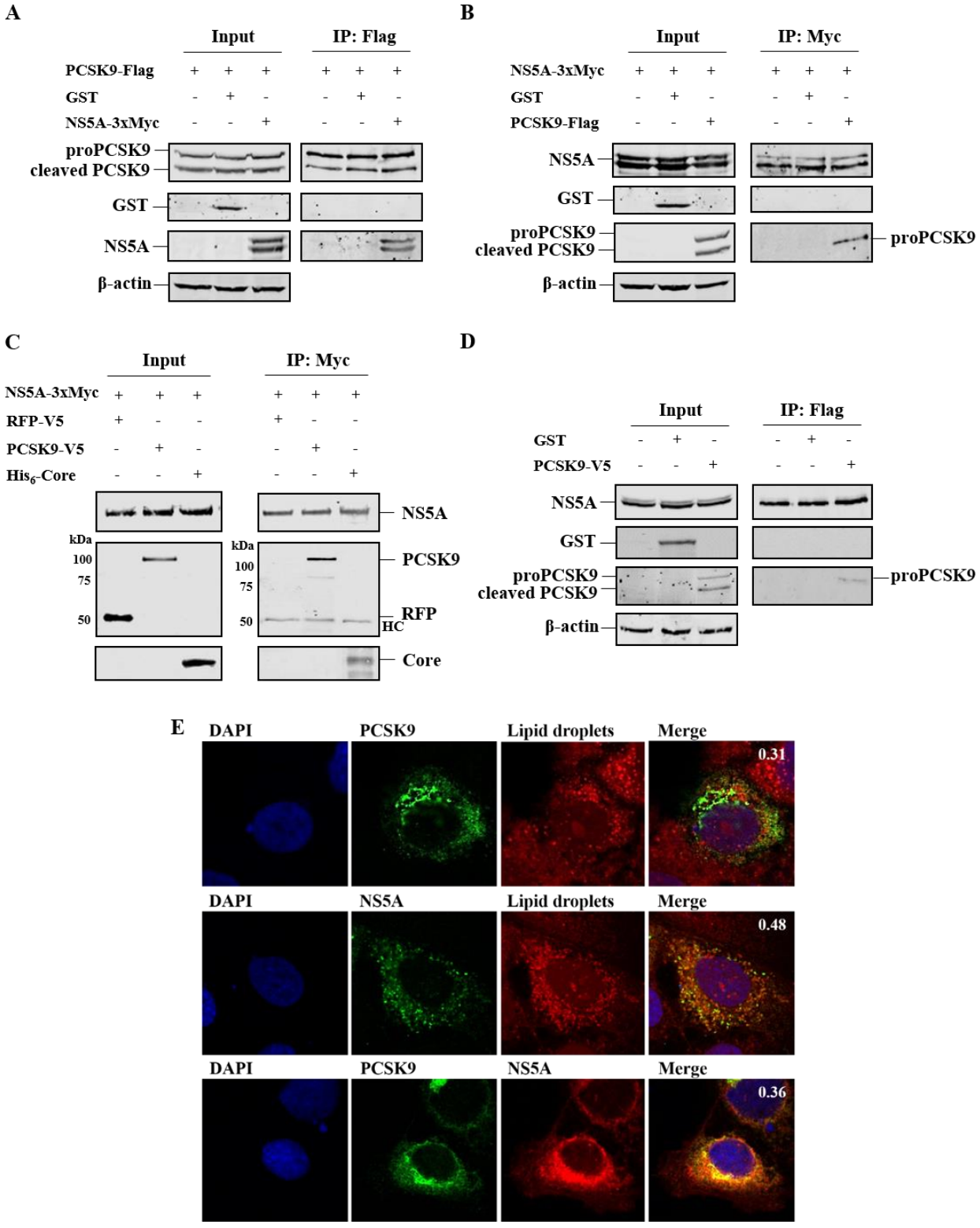

A
Input
IP: Flag
PCSK9-Flag + + + + + +
GST - + - - + -
NS5A-3xMyc - - + - - +
proPCSK9
cleaved PCSK9
GST
NS5A
β-actin
B
Input
IP: Myc
NS5A-3xMyc + + + + + +
GST - + - - + -
PCSK9-Flag - - + - - +
NS5A
GST
proPCSK9
cleaved PCSK9
β-actin
proPCSK9
C
Input
IP: Myc
NS5A-3xMyc + + + + + +
RFP-V5 + - - + - -
PCSK9-V5 - + - - + -
His6-Core - - + - - +
kDa
100
75
50
NS5A
PCSK9
RFP
HC
Core
D
Input
IP: Flag
GST - + - - + -
PCSK9-V5 - - + - - +
NS5A
GST
proPCSK9
cleaved PCSK9
β-actin
proPCSK9
E
DAPI
PCSK9
Lipid droplets
Merge
0.31
DAPI
NS5A
Lipid droplets
Merge
0.48
DAPI
PCSK9
NS5A
Merge
0.36

Figure 3.5 PCSK9 interacts and co-localizes with NS5A. HEK293T cells were co-transfected with plasmids expressing Flag-tagged PCSK9 and vector, GST or Myc-tagged NS5A (**A**), or Myc-tagged NS5A and vector, GST or Flag-tagged PCSK9 (**B**). After 48 h, cell lysates were immunoprecipitated with Flag (**A**) or Myc (**B**) antibodies and the eluted samples were subjected to Western blotting with Flag, Myc, GST and β-actin antibodies. **C**, Purified His_6-tagged HCV NS5A-Myc, PCSK9-V5, RFP-V5 and HCV core proteins were used in the co-IP assay with a Myc antibody. Eluted samples were subjected to Western blotting with Myc, V5 and His_6 antibodies. HC, heavy chain. **D**, Huh-7-RLuc-HCV-2a JFH-1 NS3-$NS5A^{Flag}$-NS5B SGR cells were transfected with plasmids expressing vector, GST or V5-tagged PCSK9. After 48 h, cell lysates were immunoprecipitated with a Flag antibody and the eluted samples were subjected to Western blotting with Flag, GST, V5 and β-actin antibodies. **E**, Huh-7 cells in chamber slides were co-transfected with plasmids expressing Flag-tagged PCSK9 and Myc-tagged NS5A. After 48 h, cells were fixed and stained with tag-specific antibodies. Nuclei were stained with DAPI and lipid droplets stained with Oil Red O. Cells were visualized using a confocal microscope. Pearson's correlation coefficients of PCSK9 and lipid droplets, NS5A and lipid droplets, or PCSK9 and NS5A were indicated in the upper-right corner of the merged pictures.

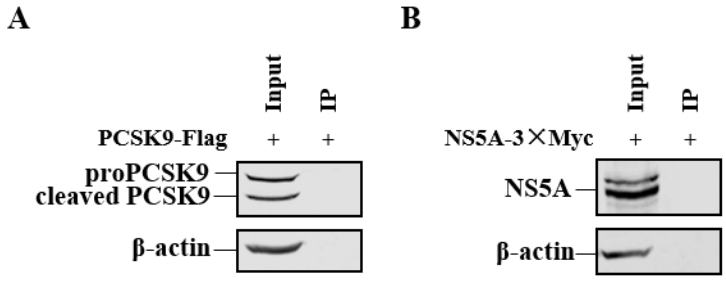


Figure 3.S5 PCSK9 or NS5A cannot bind to Dynabeads Protein G. HEK293T cells were transfected with plasmids expressing Flag-tagged PCSK9 (**A**) or Myc-tagged NS5A (**B**). After 48 h, cell lysates were incubated with Dynabeads Protein G and the eluted samples were subjected to Western blotting with Flag, Myc and β-actin antibodies.

was immunoprecipitated by NS5A (Figure 3.S6). These results suggested that NS5A preferentially interacted with proPCSK9.

To investigate whether PCSK9 co-localized with NS5A, Huh-7 cells were co-transfected with plasmids expressing PCSK9 and NS5A, and the subcellular localization was analyzed by an immunofluorescence assay. Cell nuclei and lipid droplets were also stained. As shown in Figure 3.5E, both PCSK9 and NS5A proteins localized in the cytoplasm with substantial overlap with the lipid droplet staining. More importantly, co-localization of PCSK9 and NS5A could be detected.

Taken together, these results indicated that PCSK9 directly interacted and co-localized with HCV NS5A.

3.6.7 Mapping NS5A amino acid sequences mediating PCSK9 interaction

NS5A consists of an N-terminal amphipathic helix (AH) and three structural domains I, II and III that are separated by low-complexity sequences (LCSs) (Figure 3.6A) (Moradpour and Penin, 2013). To determine which NS5A domain was involved in the interaction with PCSK9, we constructed plasmids expressing different domain truncated NS5A mutants and performed the co-IP assay. PCSK9 could interact with ΔAH NS5A, domain I-LCS I, domain I, AH-domain I-LCS I, but not with domain II-LCS II, domain II or domain III (Figure 3.6B), which suggested that domain I (aa. 35-215) was responsible for PCSK9 binding. Then we investigated the region within domain I that could interact with PCSK9. We constructed plasmids expressing different truncated NS5A domain I mutants and used them in the co-IP assay. Results showed that the full domain I (aa. 35-215) or aa. 95-215 could, while aa. 155-215 could not, bind to PCSK9 (Figure 3.6C). These results led us to speculate that aa. 95-154 of domain I was the binding region for PCSK9. In efforts to confirm this assumption, we generated plasmids expressing aa. 95-154 or aa. 155-215 and performed the co-IP assay. Interestingly, neither protein could be immunoprecipitated by PCSK9, although the input proteins were readily detectable (Figure 3.6D). These results indicated that NS5A domain I aa. 95-215 was essential for PCSK9 binding. In order to verify this finding in the context of full-length NS5A, co-IP assay was performed using plasmids that had same deletions as in domain I. The co-IP results showed that full-length NS5A or NS5A with aa. 35-94 deletion could interact with PCSK9, but NS5A with aa. 95-215 deletion

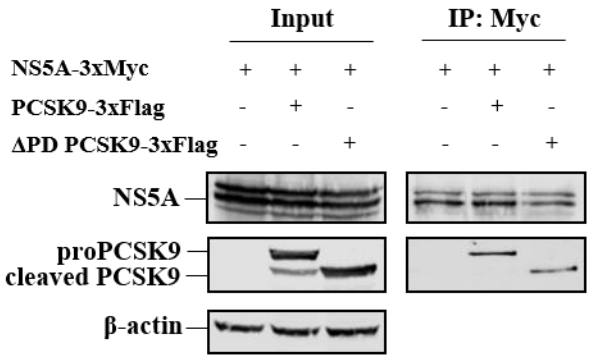
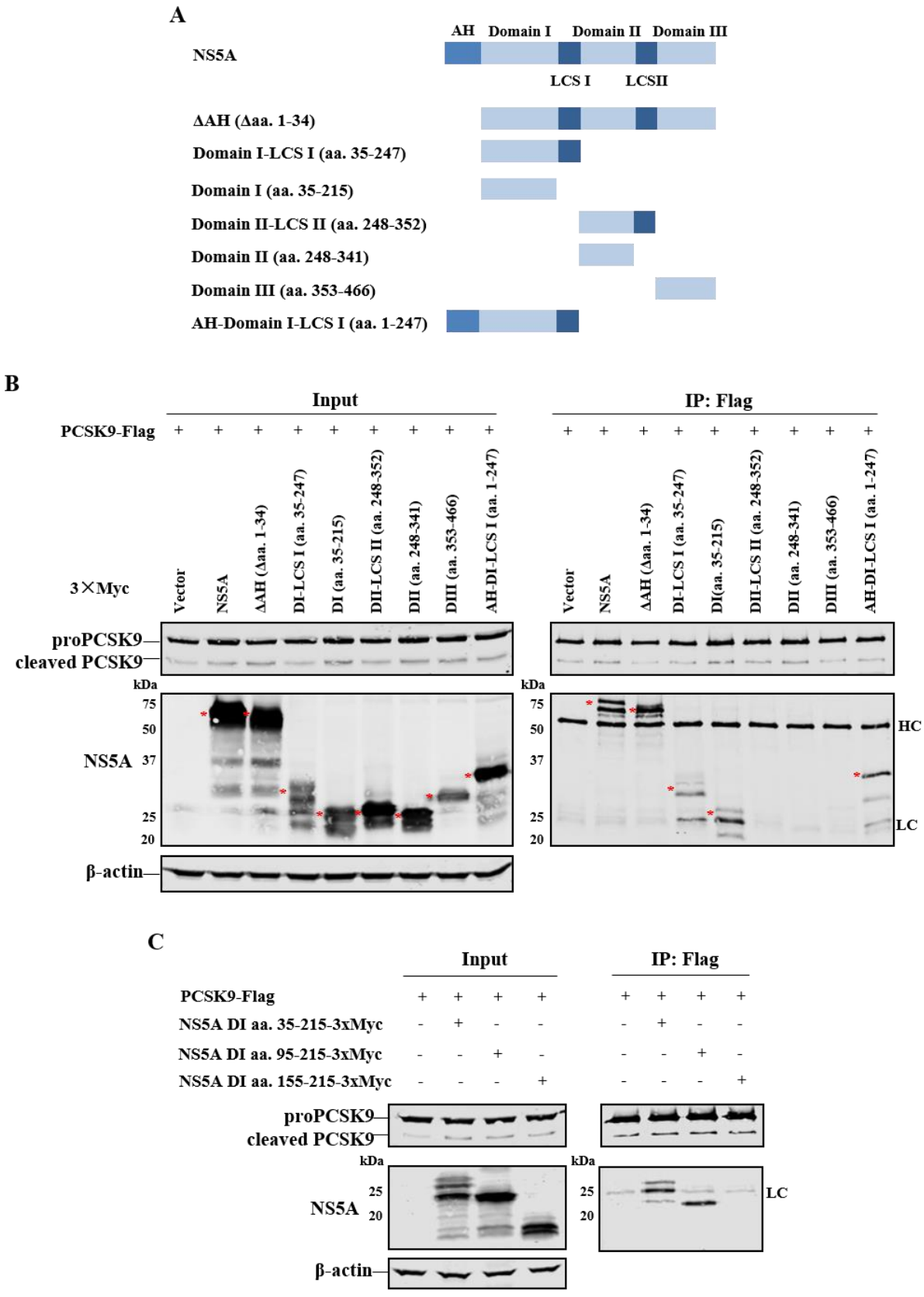


Figure 3.S6 NS5A preferentially interacts with proPCSK9. HEK293T cells were co-transfected with plasmids expressing Myc-tagged NS5A and vector, Flag-tagged wild-type, or ΔPD PCSK9. After 48 h, cell lysates were immunoprecipitated with a Myc antibody. Eluted samples were subjected to Western blotting with Myc, Flag and β-actin antibodies.


A
AH
Domain I
Domain II
Domain III
NS5A
LCS I
LCSII
ΔAH (Δaa. 1-34)
Domain I-LCS I (aa. 35-247)
Domain I (aa. 35-215)
Domain II-LCS II (aa. 248-352)
Domain II (aa. 248-341)
Domain III (aa. 353-466)
AH-Domain I-LCS I (aa. 1-247)
B
Input
IP: Flag
PCSK9-Flag
3×Myc
Vector
NS5A
ΔAH (Δaa. 1-34)
DI-LCS I (aa. 35-247)
DI (aa. 35-215)
DII-LCS II (aa. 248-352)
DII (aa. 248-341)
DIII (aa. 353-466)
AH-DI-LCS I (aa. 1-247)
proPCSK9
cleaved PCSK9
kDa
75
50
37
25
20
NS5A
HC
LC
β-actin
C
Input
IP: Flag
PCSK9-Flag
NS5A DI aa. 35-215-3xMyc
NS5A DI aa. 95-215-3xMyc
NS5A DI aa. 155-215-3xMyc
proPCSK9
cleaved PCSK9
kDa
25
20
NS5A
LC
β-actin

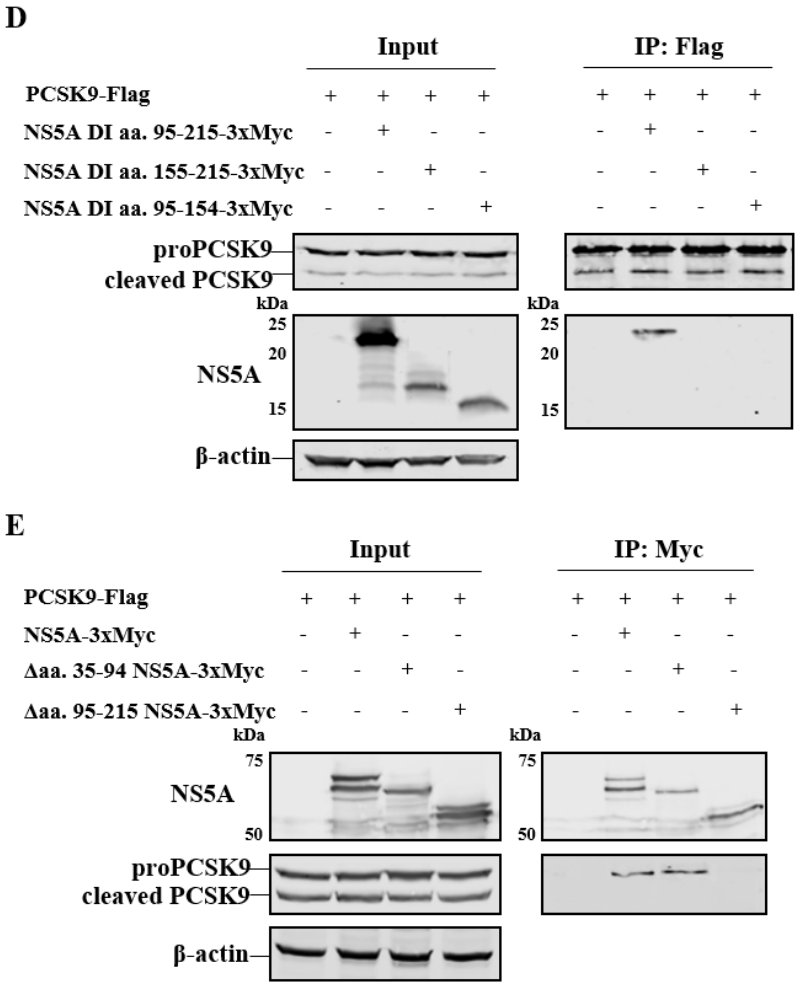
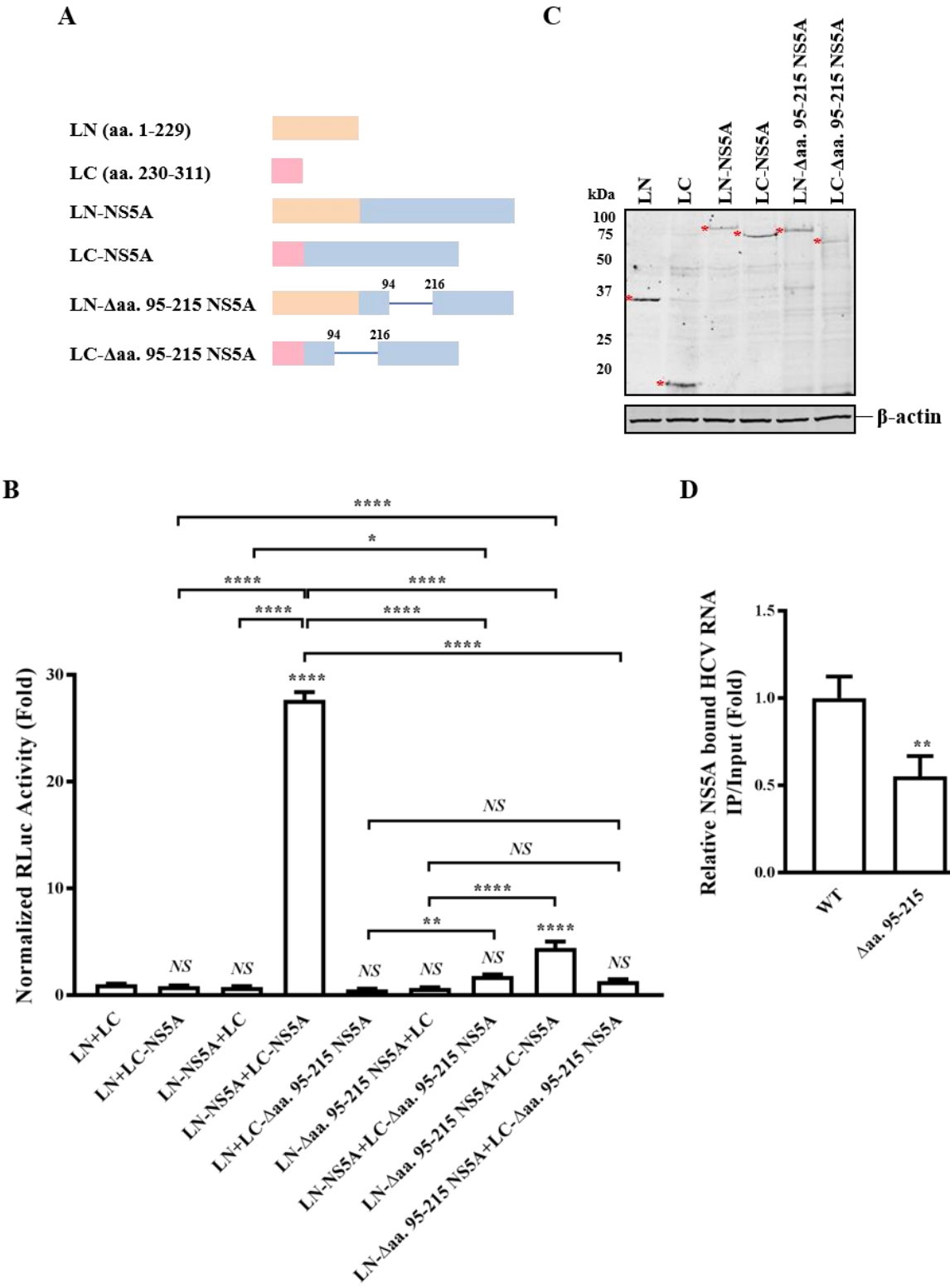


Figure 3.6 NS5A aa. 95-215 is essential for PCSK9 binding. A, The schematic diagram of NS5A domain structure and different domain truncated NS5A mutants. HEK293T cells were co-transfected with plasmids expressing Flag-tagged PCSK9 and Myc-tagged different domain truncated NS5A mutants (**B**), Myc-tagged different truncated NS5A domain I mutants (**C** and **D**), or Myc-tagged full-length NS5A that had truncations within domain I (**E**). After 48 h, cell lysates were immunoprecipitated with Flag (**B**, **C** and **D**) or Myc antibodies (**E**). Eluted samples were subjected to Western blotting with Flag, Myc and β-actin antibodies. Bands of NS5A mutants were indicated by * in (B). HC, heavy chain; LC, light chain.

could not interact with PCSK9 (Figure 3.6E). These results collectively indicated that PCSK9 interacted with NS5A through aa. 95-215 in domain I.

3.6.8 NS5A aa. 95-215 plays an important role in NS5A dimerization, NS5A-RNA binding and HCV replication

A crystal structure study of HCV-1b (Con1) NS5A domain I revealed that the NS5A dimer interface is composed of residues 92-99, 112-116, 139-143, 146-149 and 160-161 and half of these contact residues are highly conserved (Love et al., 2009). Crystallization of HCV-1a (H77) NS5A domain I showed two dimeric forms with residues 97-99, 112-115, 149 and 160-161, or 74-78 and 83-84 in the dimer contact surface (Lambert et al., 2014). Four conserved cysteines (Cys39, Cys57, Cys59 and Cys80) that consist of zinc-binding motif are also critical for HCV-1b (Con1) NS5A dimer formation (Lim et al., 2012). Since the majority of these reported essential residues for NS5A dimerization are within aa. 95-215, we speculated that HCV-2a NS5A aa. 95-215 was also essential for NS5A dimerization. To test this hypothesis, we carried out SLCA to study NS5A dimerization. SLCA is based on reconstituted luciferase activity of non-functional N- and C-terminal fragments of luciferase by fusing these fragments to interacting proteins. Protein-protein interaction can bring these two fragments of luciferase in proximity to restore the enzymatic activity (Deng et al., 2011). We constructed plasmids expressing fusion proteins of aa. 1-229 (LN) or aa. 230-311 (LC) of Renilla luciferase with full-length NS5A or NS5A with aa. 95-215 deletion (Figure 3.7A). SLCA results showed that the LN-NS5A/LC-NS5A pair exhibited a more than 26-fold increase in luciferase activity compared to controls (LN/LC, LN/LC-NS5A and LN-NS5A/LC pairs), which validated the SLCA approach for studying NS5A dimerization (Figure 3.7B). More importantly, although the LN-Δaa. 95-215 NS5A/LC-NS5A pair showed an approximately three-fold increase in luciferase activity compared to controls (LN/LC, LN/LC-NS5A and LN-Δaa. 95-215 NS5A/LC pairs), the luciferase activity of LN-NS5A/LC-Δaa. 95-215 NS5A and LN-Δaa. 95-215 NS5A/LC-Δaa. 95-215 NS5A pairs did not have a significant difference compared to their corresponding controls (Figure 3.7B). When Δaa. 95-215 NS5A was used in SLCA, the luciferase activities were reduced to the basal level with the exception of the LN-Δaa. 95-215 NS5A/LC-NS5A pair that showed an approximately three-fold increase (Figure 3.7B). Protein expression was demonstrated by Western blotting (Figure 3.7C). These results indicated an important role of aa. 95-215 in NS5A dimerization.


A
LN (aa. 1-229)
LC (aa. 230-311)
LN-NS5A
LC-NS5A
LN-Δaa. 95-215 NS5A
LC-Δaa. 95-215 NS5A
94
216
94
216
C
kDa
LN
LC
LN-NS5A
LC-NS5A
LN-Δaa. 95-215 NS5A
LC-Δaa. 95-215 NS5A
100
75
50
37
25
20
β-actin
B
Normalized RLuc Activity (Fold)
30
20
10
0
****
*
****
****
****
****
****
****
NS
NS
NS
NS
NS
NS
**
****
****
NS
NS
LN+LC
LN+LC-NS5A
LN-NS5A+LC
LN-NS5A+LC-NS5A
LN+LC-Δaa. 95-215 NS5A
LN-Δaa. 95-215 NS5A+LC
LN-Δaa. 95-215 NS5A
LN-NS5A+LC-Δaa. 95-215 NS5A
LN-Δaa. 95-215 NS5A+LC-NS5A
LN-Δaa. 95-215 NS5A+LC-Δaa. 95-215 NS5A
D
Relative NS5A bound HCV RNA IP/Input (Fold)
1.5
1.0
0.5
0.0
**
WT
Δaa. 95-215

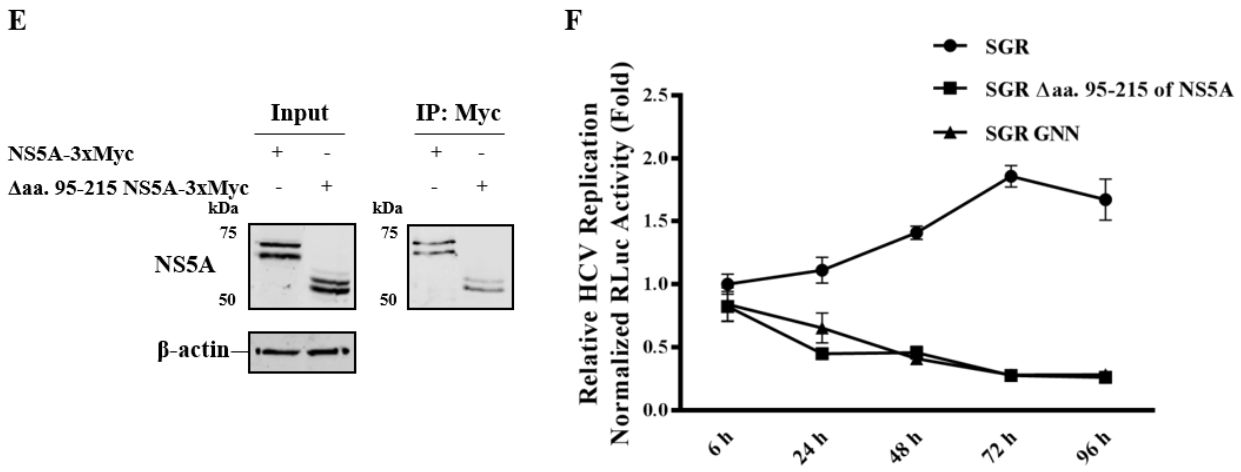


Figure 3.7 NS5A aa. 95-215 plays an important role in NS5A dimerization, NS5A-RNA binding and HCV replication. A, The schematic diagram of plasmids used in SLCA to determine the degree of NS5A dimerization. **B**, Huh-7 cells were co-transfected with indicated pairs of SLCA plasmids. Luciferase assay was performed 48 h after transfection. **C**, Huh-7 cells were transfected with indicated plasmids. Cell lysates collected 48 h after transfection were subjected to Western blotting with Myc and β-actin antibodies. Bands of Myc-tagged RLuc and NS5A proteins were indicated by *. **D**, HCV-2a J6/JFH-1(p7-RLuc2A) genomic replicon cells were transfected with plasmids expressing Myc-tagged wild-type NS5A or Myc-tagged Δaa. 95-215 NS5A. After 48 h, cell lysates were immunoprecipitated with a Myc antibody. HCV RNA levels in the immunoprecipitates were determined by RT-qPCR and plotted versus input RNA. **E**, Eluted samples from (D) were subjected to Western blotting with Myc and β-actin antibodies. **F**, Huh-7 cells were transfected with SGR RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B, RLuc-HCV-2a JFH-1 NS3-Δaa. 95-215NS5A^{Flag}-NS5B, or RLuc-HCV-2a JFH-1 NS3-NS5A^{Flag}-NS5B GNN RNAs. Luciferase assay was performed 6, 24, 48, 72 and 96 h after transfection, respectively. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, **** if $p < 0.0001$, or *NS* for not significant.

Lim et al. demonstrated that NS5A dimerization is required for its RNA binding activity as well as HCV replication (Lim et al., 2012). Therefore, we investigated whether NS5A with aa. 95-215 deletion affected NS5A-RNA binding or HCV replication. NS5A-RNA binding was determined by an RIP assay as per an established protocol (Nag et al., 2012). HCV-2a J6/JFH-1(p7-RLuc2A) genomic replicon cells were transfected with plasmids expressing Myc-tagged wild-type NS5A or Myc-tagged Δaa. 95-215 NS5A. RT-qPCR assay demonstrated that significantly less HCV RNA was immunoprecipitated by Δaa. 95-215 NS5A compared to wild-type NS5A (Figure 3.7D), which suggested that NS5A-RNA binding was decreased in the absence of aa. 95-215. To demonstrate the specificity of the RIP assay, we also measured GUSB RNA levels in immunoprecipitated RNA, which were not detectable (data not shown). Figure 3.7E confirmed the presence of Myc-tagged NS5A proteins in the immunoprecipitates.

Next, we examined the effect of NS5A with aa. 95-215 deletion on HCV replication. We transfected Huh-7 cells with HCV SGR reporter RNA with the aa. 95-215 of NS5A deleted (RLuc-HCV-2a JFH-1 NS3-Δaa. 95-215 $NS5A^{Flag}$-NS5B), and performed luciferase assay in a time course. Wild-type and replication-deficient HCV SGR RNAs were used as positive and negative controls, respectively. Figure 3.7F showed that HCV SGR RNA with NS5A aa. 95-215 deletion exhibited similar luciferase activities as the replication-deficient SGR RNA at all time points, suggesting NS5A aa. 95-215 was essential for HCV replication.

Taken together, these results indicated that aa. 95-215 of NS5A was involved in NS5A dimerization, NS5A-RNA binding, and essential for HCV replication.

3.6.9 NS5A dimerization and NS5A-RNA binding are inhibited through the interaction with PCSK9

We next investigated whether the interaction between PCSK9 and NS5A affected NS5A dimerization and NS5A-RNA binding, thereby inhibiting HCV replication. To determine the effect of PCSK9-NS5A interaction on NS5A dimerization, SLCA was carried out by co-transfecting plasmids expressing Flag-tagged PCSK9, LN-NS5A and LC-NS5A. Plasmid vector and GST-expressing plasmid were used as negative controls. Figure 3.8A showed that PCSK9 expression significantly reduced NS5A dimerization. In contrast, PCSK9 had no effect on the luciferase activity after LN-Δaa. 95-215 NS5A/LC-NS5A co-transfection (Figure 3.8B). To test the effect of PCSK9-NS5A interaction on RNA binding activity of NS5A, we performed the RIP

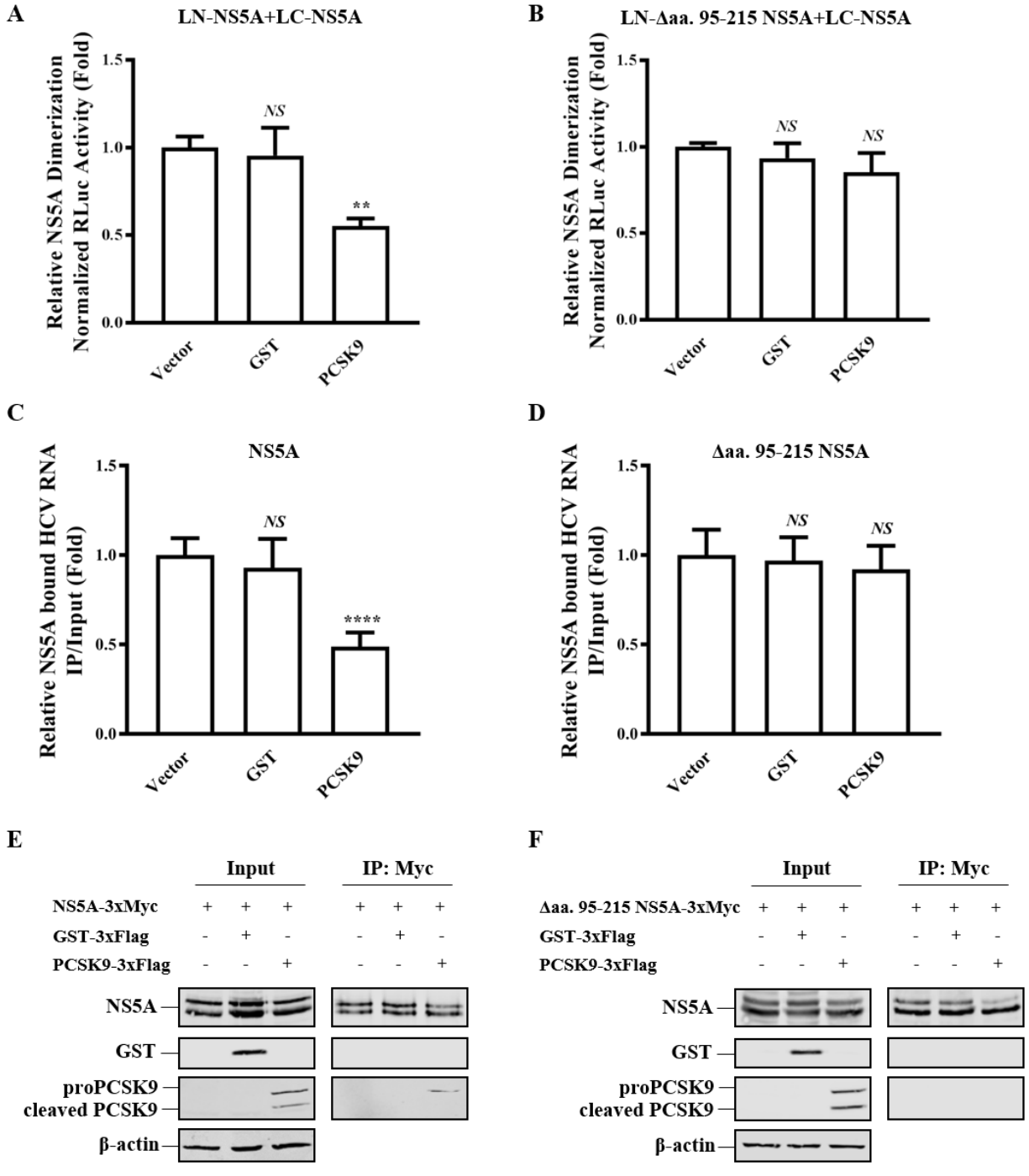

A
LN-NS5A+LC-NS5A
Relative NS5A Dimerization
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
NS
**
Vector
GST
PCSK9
B
LN-Δaa. 95-215 NS5A+LC-NS5A
Relative NS5A Dimerization
Normalized RLuc Activity (Fold)
1.5
1.0
0.5
0.0
NS
NS
Vector
GST
PCSK9
C
NS5A
Relative NS5A bound HCV RNA
IP/Input (Fold)
1.5
1.0
0.5
0.0
NS
****
Vector
GST
PCSK9
D
Δaa. 95-215 NS5A
Relative NS5A bound HCV RNA
IP/Input (Fold)
1.5
1.0
0.5
0.0
NS
NS
Vector
GST
PCSK9
E
Input
IP: Myc
NS5A-3xMyc + + + + + +
GST-3xFlag - + - - + -
PCSK9-3xFlag - - + - - +
NS5A
GST
proPCSK9
cleaved PCSK9
β-actin
F
Input
IP: Myc
Δaa. 95-215 NS5A-3xMyc + + + + + +
GST-3xFlag - + - - + -
PCSK9-3xFlag - - + - - +
NS5A
GST
proPCSK9
cleaved PCSK9
β-actin

Figure 3.8 NS5A dimerization and RNA-binding activity of NS5A are suppressed through the interaction with PCSK9. A and **B**, Huh-7 cells were co-transfected with plasmids expressing Flag-tagged PCSK9, and LN-NS5A and LC-NS5A (A), or LN-Δaa. 95-215 NS5A and LC-NS5A (**B**). Luciferase assay was performed 48 h after transfection. **C**, **D**, **E** and **F**, HCV-2a J6/JFH-1(p7-RLuc2A) genomic replicon cells were co-transfected with plasmids expressing Flag-tagged PCSK9 and Myc-tagged wild-type NS5A (**C** and **E**), or Myc-tagged Δaa. 95-215 NS5A (**D** and **F**). After 48 h, cell lysates were immunoprecipitated with a Myc antibody. HCV RNA levels in the immunoprecipitates were determined by RT-qPCR and plotted versus input RNA (**C** and **D**). Eluted samples were subjected to Western blotting with Myc, Flag and β-actin antibodies (**E** and **F**). Statistical differences between samples were demonstrated as follows: ** if $p < 0.01$, **** if $p < 0.0001$, or *NS* for not significant.

assay by co-transfecting HCV-2a J6/JFH-1(p7-RLuc2A) genomic replicon cells with plasmids expressing Flag-tagged PCSK9, and Myc-tagged NS5A, wild-type or Δaa. 95-215. Plasmid vector and GST-expressing plasmid were used as negative controls. In comparison to vector or GST, expression of PCSK9 resulted in significantly less HCV RNA in the Myc immunoprecipitates, suggesting that the RNA-binding activity of NS5A was suppressed in the presence of PCSK9 (Figure 3.8C). In contrast, there was no significant difference in immunoprecipitated HCV RNA levels among cells co-transfected with plasmids expressing Δaa. 95-215 NS5A and vector, GST or PCSK9 (Figure 3.8D). Figure 3.8E and F confirmed the interaction between PCSK9 and NS5A through aa. 95-215. These results indicated that the interaction between PCSK9 and NS5A inhibited NS5A dimerization and HCV RNA binding to NS5A, and PCSK9 no longer affected these NS5A properties when the interaction was disrupted.

3.7 Discussion

The antiviral effect of PCSK9 on HCV has not been thoroughly characterized. Given the tremendous interests of using PCSK9 as a drug target in a variety of clinical conditions, we further studied the effect of PCSK9 on the HCV life cycle.

PCSK9 is actively involved in modulating lipid homeostasis (Melendez et al., 2017). A previous study from our group showed that sterol regulatory element-binding proteins, transcription factors for lipogenesis, regulate HCV RNA translation (Shi et al., 2016). HCV virion assembly and secretion are also closely connected with the host lipoprotein assembly and secretion pathways (Syed et al., 2010). One would therefore assume a possible role for PCSK9 in these processes. Interestingly, however, our results showed that PCSK9 did not affect HCV translation, virion assembly or secretion (Figure 3.1 and 3.S1). These may suggest that the pathways regulating HCV translation and assembly/secretion are separate from those modulated by PCSK9.

Although PCSK9 has been demonstrated to down-regulate HCV replication, the molecular mechanisms are not known. We therefore focused our subsequent study on this aspect. We first demonstrated that PCSK9 impeded HCV replication in genomic replicon cells in a dose-dependent manner (Figure 3.2B and 3.S2B). Furthermore, by shRNA knockdown and in combination with expressing an shRNA-resistant PCSK9, we demonstrated the specificity of the observed changes in HCV replication (Figure 3.3 and 3.S3). We also showed that PCSK9

inhibited HCV replication after HCV infection (Figure 3.2D). These results reinforced and extended previously recognized inhibitory effects of PCSK9 on HCV replication.

Because of the role of PCSK9 in LDLR degradation and the importance of LDLR in HCV replication, we reasoned that LDLR degradation might be involved in PCSK9-induced HCV replication inhibition. To our surprise, neither gain-of-function mutant nor loss-of-function mutant of PCSK9 showed a significant difference in HCV replication inhibition compared to wild-type PCSK9 (Figure 3.2E, G and 3.S2C). These results suggested that the inhibitory effect of PCSK9 on HCV replication was independent of LDLR degradation. Our data extended a previous study by also including a loss-of-function mutant of PCSK9 (Syed et al., 2014).

Both proPCSK9 and cleaved PCSK9 are present in cells. We showed only proPCSK9 could regulate HCV replication and cleaved PCSK9 did not (Figure 3.4 and 3.S4). On the other hand, increasing the levels of cleaved PCSK9 was associated with decreasing auto-cleavage of the proPCSK9 and thus resulting in increased proPCSK9 levels (Figure 3.4D). It implied a negative feedback loop between proPCSK9 and cleaved PCSK9. The underlying mechanism for the differential effects on regulating HCV replication by two PCSK9 forms is not clear. An interesting hypothesis would be that the proteolytic cleavage of PCSK9 is involved in regulating HCV replication that should be studied further. This mode of action is not unprecedented since La protein cleavage has been shown to play a role in regulating HCV (Romero et al., 2009).

Since protein-protein interaction is one of the mechanisms for PCSK9 to exert its functions, we hypothesized that PCSK9 might interact with HCV proteins to regulate HCV replication. Considering that NS5A has been shown to interact with numerous cellular proteins and many of which play a role in HCV replication, we focused on NS5A. Indeed, we discovered that PCSK9 directly interacted and co-localized with NS5A (Figure 3.5). Moreover, we demonstrated an interaction between PCSK9 and NS5A in HCV-replicating cells (Figure 3.5D). We would like to point out that these experiments were performed after ectopic expression of PCSK9. Our attempts to demonstrate an interaction between NS5A and endogenous PCSK9 were not successful probably because the endogenous PCSK9 level was too low to be detected in Western blotting after immunoprecipitation (data not shown).

We next identified that NS5A domain I aa. 95-215 was essential to interact with PCSK9 (Figure 3.6). NS5A domain I contributes to NS5A dimerization and RNA binding, which is a determinant for HCV replication (Hwang et al., 2010; Moradpour and Penin, 2013). Therefore,

we studied the role of NS5A domain I aa. 95-215 in NS5A biological functions. Figure 3.7 showed aa. 95-215 deletion impaired NS5A dimerization, NS5A-RNA binding and had a lethal effect on HCV replication. More importantly, we demonstrated that PCSK9 decreased NS5A dimerization and NS5A-RNA binding (Figure 3.8). However, when the interaction between PCSK9 and NS5A was interrupted by deleting NS5A aa. 95-215, PCSK9 no longer affected these properties (Figure 3.8). Our results strongly suggested that HCV replication inhibition by PCSK9 could result from disrupting NS5A dimerization and NS5A-RNA binding through protein-protein interaction.

A previous study showed that HCVcc-2a infection is associated with reduced intracellular PCSK9 levels in Huh-7 cells (Syed et al., 2014). Taken the inhibitory effect of PCSK9 on HCV replication into consideration, it seems logical for HCV to down-regulate PCSK9 level. However, clinical studies have demonstrated a much more complex picture. It has been reported that significantly higher plasma PCSK9 concentrations are measured in HCV-1 patients compared to HCV negative population, while plasma PCSK9 concentrations in HCV-3 patients are significantly lower. Moreover, there is no correlation between PCSK9 concentration and total cholesterol or LDLC level in HCV-1 or HCV-3 patients, whereas such a correlation is shown in HCV negative population (Bridge et al., 2015). It indicates that HCV disrupts LDLC homeostasis and PCSK9 expression as well as plasma PCSK9 concentration in a genotype-specific manner. HCV treatment is more effective in patient with high plasma LDLC level (Gopal et al., 2006). Given the role of PCSK9 in inhibiting HCV entry and replication and increasing plasma LDLC level, elevating PCSK9 level can be considered as a means to improve the efficacy of treating HCV infection. However, high PCSK9 level is associated with hypercholesterolemia and PCSK9 concentrations are different according to HCV genotypes. As such, PCSK9 may be selectively used for anti-HCV treatment according to the infecting genotype, and the antiviral effect and hypercholesterolemia of PCSK9 should be balanced.

In summary, we have demonstrated that PCSK9 did not affect HCV translation, virion assembly or secretion. PCSK9 inhibited HCV replication in a dose-dependent manner. The interaction between NS5A and PCSK9 suppressed NS5A dimerization and RNA-binding activity of NS5A, which might contribute to HCV replication inhibition. This study should improve the understanding of the antiviral effect of PCSK9 on HCV and help optimize anti-HCV regimens.

3.8 Acknowledgements

We would like to thank Dr. Charles Rice for providing Huh-7.5 cells and HCV-2a J6/JFH-1 p7-RLuc2A plasmids, Drs. Daping Fan and Thomas Lagace for providing PCSK9 plasmids, Dr. Feng Li for providing split luciferase complementation assay plasmids. We thank Guanqun Liu for helpful discussions. This work was supported by grants from Canadian Institutes of Health Research, Saskatchewan Health Research Foundation, and Natural Sciences and Engineering Research Council of Canada to QL. ZL is a recipient of a University of Saskatchewan Vaccinology and Immunotherapeutics Graduate Student scholarship. This article is published with the permission of the Director of VIDO-InterVac, journal series no. 811.

4.0 LINKER BETWEEN CHAPTERS 3.0 AND 5.0

In Chapter 3.0, I studied the first objective that is to investigate the role of PCSK9 in different stages of the HCV life cycle. I demonstrated that PCSK9 can inhibit HCV replication and has no effect on HCV translation, virion assembly or secretion. Then I investigated how PCSK9 down-regulates HCV replication and found that it does not result from LDLR degradation induced by PCSK9. I also showed that the interaction between PCSK9 and NS5A that disrupts NS5A dimerization and NS5A and HCV-RNA binding leads to the inhibition of HCV replication.

Except for interacting with NS5A, PCSK9 may use different strategies to inhibit HCV replication. I intended to find out if there are other mechanisms. Since IFN plays an important role in viral clearance (Schoggins and Rice, 2013), I hypothesized that PCSK9 suppresses HCV replication via elevating IFN expression. Therefore, I examined the effect of PCSK9 on IFN expression in Chapter 5.0.

5.0 PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE INHIBITS INTERFERON β EXPRESSION THROUGH INTERACTING WITH ATF-2

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Keywords: ATF-2/c-Jun complex; IFNβ expression; PCSK9

Running title: **PCSK9 inhibits IFNβ expression**

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5.2 Authors' Contribution

All the experiments within this chapter were performed by Zhubing Li. The manuscript was written by Zhubing Li and edited by Qiang Liu.

5.3 Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) regulates lipid metabolism. A complex interplay of lipid homeostasis and innate immune system has been increasingly recognized. We therefore studied the effect of PCSK9 on interferon (IFN) β expression. We show that PCSK9 decreases IFNβ promoter/enhancer activity, mRNA and protein levels, and its downstream 2',5'-oligoadenylate synthetase-1 mRNA level. ProPCSK9, but not the cleaved PCSK9, down-regulates IFNβ promoter/enhancer activity. Moreover, PCSK9 decreases IFNβ promoter/enhancer activity through the positive regulatory domain IV region where the activating transcription factor-2 (ATF-2)/c-Jun heterodimer binds. Mechanistically, we demonstrate an interaction between PCSK9 and ATF-2, which reduces ATF-2/c-Jun dimerization and ATF-2/c-Jun binding to the IFNβ enhancer. This novel function of PCSK9 should have important implications in optimizing the clinical use of PCSK9 inhibitors.

5.4 Introduction

Host innate immune system acts as the first line to fight against viral infections, such as hepatitis C virus (HCV) infection. Invading HCV RNA can be sensed by pathogen recognition receptors (PRRs), including toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-

like receptors, which sequentially activate downstream interferon (IFN) regulatory factor (IRF)-3 to induce type I, type III IFN and IFN-stimulated genes (ISGs) production (Chan and Ou, 2017; Heim, 2013; Shi et al., 2017). Secreted IFNs can act on neighbouring cells to activate Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway to induce transcription of numerous ISGs, resulting in HCV replication inhibition (Heim and Thimme, 2014; Schoggins and Rice, 2013). Therefore, IFN production is critical for clearance of virus infections.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is the ninth and last member in the proprotein convertase family, which is linked to familial hypercholesterolemia (Seidah et al., 2017; Seidah et al., 2003). It serves as a natural secreted inhibitor of low-density lipoprotein receptor (LDLR) by promoting lysosomal degradation of LDLR, and therefore it plays an important role in lipoprotein regulation (Gu and Zhang, 2015; Lambert et al., 2009). In addition, a recent paper demonstrated that PCSK9 expression is induced upon inhibition of the JAK/STAT pathway (Ruscica et al., 2016), suggesting a possible interplay of PCSK9 and the host innate immune system.

We and others have reported that PCSK9 can inhibit HCV entry and replication (Labonte et al., 2009; Li and Liu, 2018; Syed et al., 2014). Given the inhibitory effect of IFNs on HCV replication, we hypothesized that PCSK9 may inhibit HCV by modulating IFN production. Somewhat to our surprise, our results showed that PCSK9 down-regulated IFNβ expression, suggesting that PCSK9 does not inhibit HCV replication through modulating IFNβ. However, since inhibiting IFNβ expression represents a novel function of PCSK9, we investigated the molecular mechanisms in this study. We showed that PCSK9 suppressed IFNβ expression through inhibiting activating transcription factor-2 (ATF-2)/c-Jun dimerization and ATF-2/c-Jun binding to the IFNβ enhancer via the interaction with ATF-2.

5.5 Materials and Methods

5.5.1 Plasmids and *in vitro* transcription

Plasmid expressing V5-tagged PCSK9 was received from Dr. Daping Fan (Du et al., 2011). Plasmid expressing c-Jun was received from Dr. En-Min Li (Gao et al., 2009). Plasmid pDONR223_ATF2_WT was a gift from Drs. Jesse Boehm, Matthew Meyerson, and David Root (Addgene plasmid # 82889) (Berger et al., 2016). HCV-2a JFH-1 genomic plasmid pHCV-2a

JFH-1_pUC was received from Dr. Takaji Wakita (Wakita et al., 2005). Split luciferase complementation assay (SLCA) vectors pLC and pLN were received from Dr. Feng Li (Deng et al., 2011). Plasmids expressing Flag-tagged wild-type, Q152H and ΔPD PCSK9 were described previously (Li and Liu, 2018). The coding sequence of c-Jun was cloned into the p3xFlag-CMV-7.1 vector (Sigma-Aldrich) or the pcDNA3.1 vector (Thermo Fisher Scientific) with an N-terminal Myc-tag, respectively. The coding sequence of ATF-2 was cloned into the pcDNA3.1 vector (Thermo Fisher Scientific) with an N-terminal Myc-tag. Flag-tagged ATF-2 and Myc-tagged c-Jun were also cloned into SLCA vectors pLC or pLN (Li and Liu, 2018), respectively. Enhanced green fluorescent protein (EGFP)-expressing plasmid pEGFP-C1 (Takara Bio USA) as well as plasmid expressing EGFP with a C-terminal Flag-tag were used as controls. Human IFNβ promoter/enhancer sequences of different lengths were cloned into the pGL4.10 vector (Promega). The 3' untranslated region (3'UTR) sequence of HCV-2a JFH-1 was cloned into the pGEM 7Zf vector with a T7 promoter (Promega). For *in vitro* transcription, plasmid encoding HCV-2a JFH-1 3'UTR was linearized by *Kpn*2I (Thermo Fisher Scientific) and transcribed into RNA using MEGAscript T7 Transcription Kit (Thermo Fisher Scientific).

5.5.2 Cell lines, transfection, luciferase assay and Western blotting

Huh-7 and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich) at 37°C and 5% CO_2. Cells were transfected with DNA or co-transfected with DNA and RNA using the jetPEI reagent (Polyplus) according to the manufacturer's protocol. Cells were lysed for luciferase assay or Western blotting as previously described (Li and Liu, 2018). The primary antibodies used in Western blotting were Flag-tag (Sigma-Aldrich), AFP/EGFP (MP Biomedicals), Myc-tag, V5-tag and β-actin (Cell Signaling Technology), and secondary antibodies were IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

5.5.3 Reverse transcription quantitative real-time PCR

The total RNA of transfected Huh-7 cells was extracted and reverse transcribed into cDNA, and quantitative real-time PCR (qPCR) was performed to determine IFNβ and 2',5'-oligoadenylate synthetase (OAS)-1 RNA levels using primers IFNβ-FD-1 (5'-

AAACTCATGAGCAGTCTGCA-3'), IFNβ-Rev-1 (5'-AGGAGATCTTCAGTTTCGGAGG-3'), 2',5'-OAS-1-FD (5'-CTCAAGAGCCTCATCCG-3'), and 2',5'-OAS-1-Rev (5'-GCAGAGTTGCTGGTAGTTTA-3') as previously described (Li and Liu, 2018). The RNA levels of housekeeping gene β-glucuronidase (GUSB) were also measured using primers GUSB-FD (5'-GGTGCTGAGGATTGGCAGTG-3') and GUSB-Rev (5'-CGCACTTCCAACTTGAACAGG-3') for normalization. GUSB has been shown to be one of the most reliable housekeeping genes in liver cells including Huh-7 (Congiu et al., 2011).

5.5.4 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed using human IFN-beta bioluminescent ELISA kit according to the manufacturer's protocol (Invivogen). Briefly, the plate was prepared by coating with 1 μg/mL capture antibody that was diluted in coating buffer (0.2 M carbonate/bicarbonate buffer, pH 9.4) for overnight at room temperature and then incubating with blocking buffer (PBS, 2% BSA, 0.05% Tween 20) for 2 h at 37°C. After incubating the plate with samples for 2 h at 37°C, 10 ng/mL Lucia conjugated antibody that was diluted in reagent diluent (PBS, 1% BSA, 0.05% Tween 20) was added to the plate and incubated for 2 h at 37°C. Finally, QUANTI-Luc assay solution was added to the plate and the luciferase activity was measured immediately using VICTOR^3 V multilabel plate reader (PerkinElmer).

5.5.5 Co-immunoprecipitation and chromatin immunoprecipitation

Co-immunoprecipitation (co-IP) experiment was performed as previously described (Li and Liu, 2018). For the chromatin immunoprecipitation (ChIP) experiment, transfected Huh-7 cells were cross-linked with 1% formaldehyde for 15 min at 37°C and followed by quenching with 125 mM glycine for 5 min at 37°C. Cells were then lysed in a lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche) at 4°C. The cell lysates were digested with micrococcal nuclease (Cell Signaling Technology) for 20 min at 37°C before immunoprecipitated with indicated antibody using Dynabeads Protein G (Thermo Fisher Scientific) for 1 h at room temperature. After washing, the samples were eluted in an elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at 65°C. Then the cross-linking was reversed by incubating with 200 mM NaCl for overnight at 65°C and proteinase K for 1 h at 45°C. Finally, DNA was extracted using

phenol/chloroform and qPCR was performed using primers IFNβ-FD-2 (5'-AACATTAGAAAACCTCACAGTTTGT-3') and IFNβ-Rev-2 (5'-ATTTCCCACTTTCACTTCTCCCTT-3').

5.5.6 Statistical analysis

All experiments were performed in triplicate and data were analyzed using GraphPad Prism 7. If the *p* value determined by one- or two-way analysis of variance (ANOVA) is less than 0.05, it is considered as statistically significant.

5.6 Results

5.6.1 PCSK9 inhibits IFNβ expression

We first examined the effect of PCSK9 on IFNβ expression, a type I IFN (Snell et al., 2017). We co-transfected Huh-7 cells with HCV-2a JFH-1 3'UTR RNA and vector, or plasmids expressing EGFP or PCSK9, and determined IFNβ mRNA level by reverse transcription (RT)-qPCR and IFNβ protein level by ELISA. HCV 3'UTR contains a polyU/UC region, which is the pathogen-associated molecular pattern (PAMP) that can be recognized by PRRs to trigger IFN production (Saito et al., 2008). Our results showed that IFNβ mRNA and protein levels in cells transfected with PCSK9 were lower than that in cells transfected with vector or EGFP (Figure 5.1A and B). It indicated that PCSK9 down-regulated IFNβ expression. To demonstrate whether the intracellular IFNβ signaling was also inhibited by PCSK9 as a result of decreased IFNβ protein level, we measured 2',5'-OAS-1 mRNA level in cells co-transfected with HCV-2a JFH-1 3'UTR RNA and PCSK9. 2',5'-OAS-1 is an ISG that can be induced by type I IFN and inhibits HCV replication (Kwon et al., 2013; Metz et al., 2013). Figure 5.1C showed that 2',5'-OAS-1 mRNA level decreased in PCSK9-expressing cells compared to the control cells. The expression of EGFP and PCSK9 was demonstrated by Western blotting (Figure 5.1D). These results indicated that PCSK9 reduces IFNβ transcript and protein levels.

Since IFNβ transcription is controlled by its promoter and enhancer, we then studied the effect of PCSK9 on IFNβ promoter and enhancer activities to substantiate the above finding. We used luciferase reporter constructs with IFNβ promoter/enhancer of different lengths. The -39/+19 fragment contains the IFNβ core promoter with the TATA box and the cap site that are essential for transcription initiation, but not involved in IFNβ transcription modulation by viruses

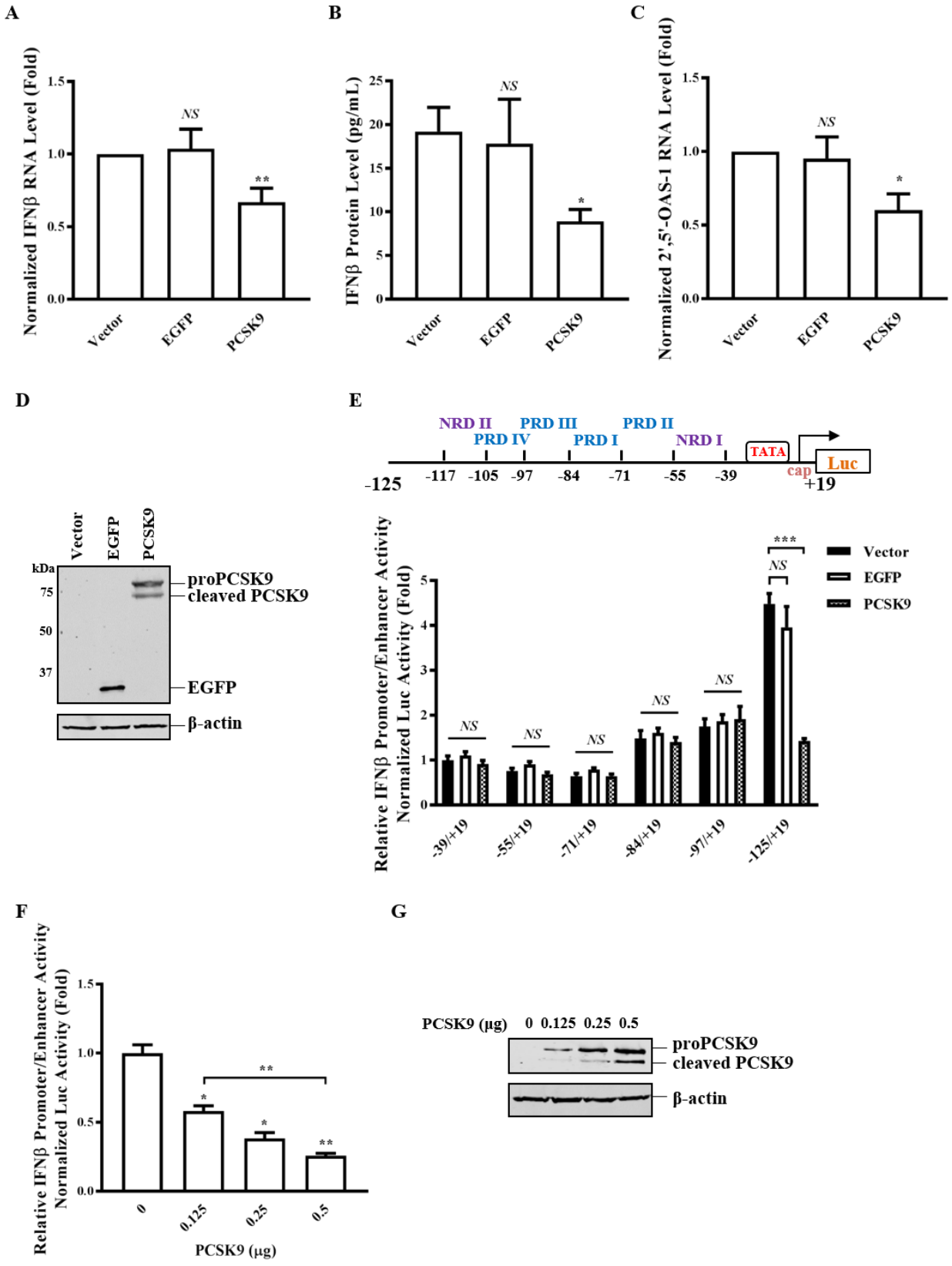
A
Normalized IFNβ RNA Level (Fold)
1.5
1.0
0.5
0.0
NS
**
Vector
EGFP
PCSK9
B
IFNβ Protein Level (pg/mL)
25
20
15
10
5
0
NS
*
Vector
EGFP
PCSK9
C
Normalized 2',5'-OAS-1 RNA Level (Fold)
1.5
1.0
0.5
0.0
NS
*
Vector
EGFP
PCSK9
D
Vector
EGFP
PCSK9
kDa
75
50
37
proPCSK9
cleaved PCSK9
EGFP
β-actin
E
NRD II
PRD III
PRD II
PRD IV
PRD I
NRD I
TATA
Luc
cap
-125
-117
-105
-97
-84
-71
-55
-39
+19
Relative IFNβ Promoter/Enhancer Activity
Normalized Luc Activity (Fold)
5
4
3
2
1
0
***
NS
NS
NS
NS
NS
NS
Vector
EGFP
PCSK9
-39/+19
-55/+19
-71/+19
-84/+19
-97/+19
-125/+19
F
Relative IFNβ Promoter/Enhancer Activity
Normalized Luc Activity (Fold)
1.5
1.0
0.5
0.0
**
*
*
**
0
0.125
0.25
0.5
PCSK9 (μg)
G
PCSK9 (μg) 0 0.125 0.25 0.5
proPCSK9
cleaved PCSK9
β-actin

Figure 5.1 PCSK9 inhibits IFNβ expression. A and **C**, Huh-7 cells were co-transfected with HCV-2a JFH-1 3'UTR RNA and vector, or plasmids expressing Flag-tagged EGFP or PCSK9. After 24 h, total RNA was extracted and RT-qPCR was performed to determine IFNβ (**A**) and 2',5'-OAS-1 (**C**) transcript levels. **B**, Huh-7 cells were co-transfected with HCV-2a JFH-1 3'UTR RNA and vector, or plasmids expressing Flag-tagged EGFP or PCSK9. After 48 h, the supernatant was subjected to ELISA. **D**, Cell lysates from (B) were subjected to Western blotting using Flag-tag and β-actin antibodies. **E**, The schematic of -125/+19 IFNβ promoter/enhancer-luciferase reporter with basic transcription elements and regulatory domains indicated. Huh-7 cells were co-transfected with different regulatory domain truncated constructs of IFNβ promoter/enhancer-luciferase reporters and vector, or plasmids expressing Flag-tagged EGFP or PCSK9. After 48 h, luciferase assay was performed. **F**, Huh-7 cells were co-transfected with -125/+19 IFNβ promoter/enhancer-luciferase reporter and increasing amounts of plasmid expressing Flag-tagged PCSK9. After 48 h, luciferase assay was performed. **G**, Cell lysates from (F) were subjected to Western blotting using Flag-tag and β-actin antibodies. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, *** if $p < 0.001$, or *NS* for not significant.

(Fujita et al., 1985). The IFNβ enhancer consists of four positive regulatory domains (PRDs) (Ford and Thanos, 2010). PRDI within DNA sequence -84 to -71 upstream of IFNβ transcription start site and PRDIII within DNA sequence -97 to -84 have the binding sites for transcription factor IRFs. PRDII (-71 to -55) can be recognized by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), but NF-κB itself is not sufficient for IFNβ expression. PRDIV within -105 to -97 contains the binding site for ATF-2/c-Jun complex (Ford and Thanos, 2010; King and Goodbourn, 1994; Maniatis et al., 1998; Moriyama et al., 2007; Yoneyama et al., 1998; Yoneyama et al., 1996). The IFNβ enhancer and its binding transcription factors form an enhanceosome that can activate IFNβ promoter (Ford and Thanos, 2010). The -125/+19 IFNβ promoter/enhancer contains the functional proximal 5' upstream sequence that can confer maximal IFNβ expression upon viral infection or other inducers (Fujita et al., 1985; Yoneyama et al., 1996). In addition, there are two negative regulatory domains (NRDs) within DNA sequence -55 to -39, -117 to -105 of the -125/+19 IFNβ promoter/enhancer, respectively. They are responsible for repressing IFNβ expression in uninfected cells (King and Goodbourn, 1994). To determine the IFNβ promoter/enhancer activity, Huh-7 cells were co-transfected with different regulatory domain truncated constructs of IFNβ promoter/enhancer-luciferase reporters and vector, or plasmids expressing EGFP or PCSK9. Figure 5.1E showed that the luciferase activity under the control of the -71/+19 IFNβ promoter/enhancer was similar to that of -39/+19 promoter and -55/+19 IFNβ promoter/enhancer in vector- and EGFP-expressing cells. It confirmed that NF-κB itself could not activate the IFNβ promoter/enhancer activity. As the IFNβ promoter/enhancer extended from -71 to -125, IFNβ promoter/enhancer activity gradually increased. In comparison to vector and EGFP expression, ectopic expression of PCSK9 had no effect on the IFNβ promoter/enhancer activities of -39/+19, -55/+19, -71/+19, -84/+19, or -97/+19 constructs (Figure 5.1E). However, PCSK9 significantly reduced luciferase activity of the -125/+19 IFNβ promoter/enhancer compared to vector or EGFP (Figure 5.1E), suggesting that PCSK9 inhibited IFNβ promoter/enhancer activity when PRDIV was present in the promoter/enhancer region. Taken together, these results indicated that PCSK9 decreases IFNβ transcription by inhibiting its promoter/enhancer activity.

To study whether PCSK9 inhibited IFNβ promoter/enhancer activity in a dose-dependent manner, we co-transfected Huh-7 cells with -125/+19 IFNβ promoter/enhancer-luciferase reporter and increasing amounts of PCSK9. Luciferase assay showed that the increasing levels of

PCSK9 resulted in more pronounced inhibition of IFNβ promoter/enhancer activity (Figure 5.1F), suggesting a dose-dependent effect of PCSK9 on down-regulating IFNβ promoter/enhancer activity. The expression of PCSK9 was confirmed by Western blotting (Figure 5.1G).

5.6.2 ProPCSK9 down-regulates IFNβ promoter/enhancer activity

PCSK9 consists of a small signal peptide, a prodomain (PD), a catalytic domain and a C-terminal domain (Farnier, 2014; Lambert et al., 2009). The PD domain is removed upon an auto-cleavage and therefore both proPCSK9 and cleaved PCSK9 are present (Figure 5.1D and G). To determine whether the inhibition of IFNβ promoter/enhancer activity was due to proPCSK9 or cleaved PCSK9, we used a PCSK9 Q152H mutant that only expresses proPCSK9 by mutating the cleavage site and ΔPD PCSK9 that only expresses cleaved PCSK9 by deleting PD in the promoter/enhancer-luciferase reporter assay. Figure 5.2A showed that Q152H PCSK9 down-regulated IFNβ promoter/enhancer activity, which was similar as wild-type PCSK9. However, ΔPD PCSK9 did not alter IFNβ promoter/enhancer activity. Figure 5.2B confirmed that Q152H PCSK9 and ΔPD PCSK9 expressed proPCSK9 and cleaved PCSK9, respectively. These results suggested that proPCSK9 inhibited IFNβ promoter/enhancer activity.

To further confirm that only proPCSK9 played a role in regulating IFNβ promoter/enhancer activity, we co-transfected Huh-7 cells with -125/+19 IFNβ promoter/enhancer-luciferase reporter, fixed amount of wild-type V5-tagged PCSK9 and increasing amounts of Flag-tagged ΔPD PCSK9. Increasing ΔPD PCSK9 resulted in down-regulating V5-tagged cleaved PCSK9 and up-regulating V5-tagged proPCSK9, suggesting that PCSK9 auto-cleavage was inhibited in the presence of cleaved PCSK9 (Figure 5.2D). Correspondingly, IFNβ promoter/enhancer activity gradually decreased with the increasing levels of proPCSK9 (Figure 5.2C). As a control, we performed the promoter/enhancer-luciferase reporter assay using increasing amounts of ΔPD PCSK9. There was no difference in IFNβ promoter/enhancer activity among cells expressing different amounts of ΔPD PCSK9 (Figure 5.2E). Western blotting confirmed the expression of ΔPD PCSK9 (Figure 5.2F).

Taken together, these results indicated that proPCSK9, but not cleaved PCSK9, down-regulates IFNβ promoter/enhancer activity.

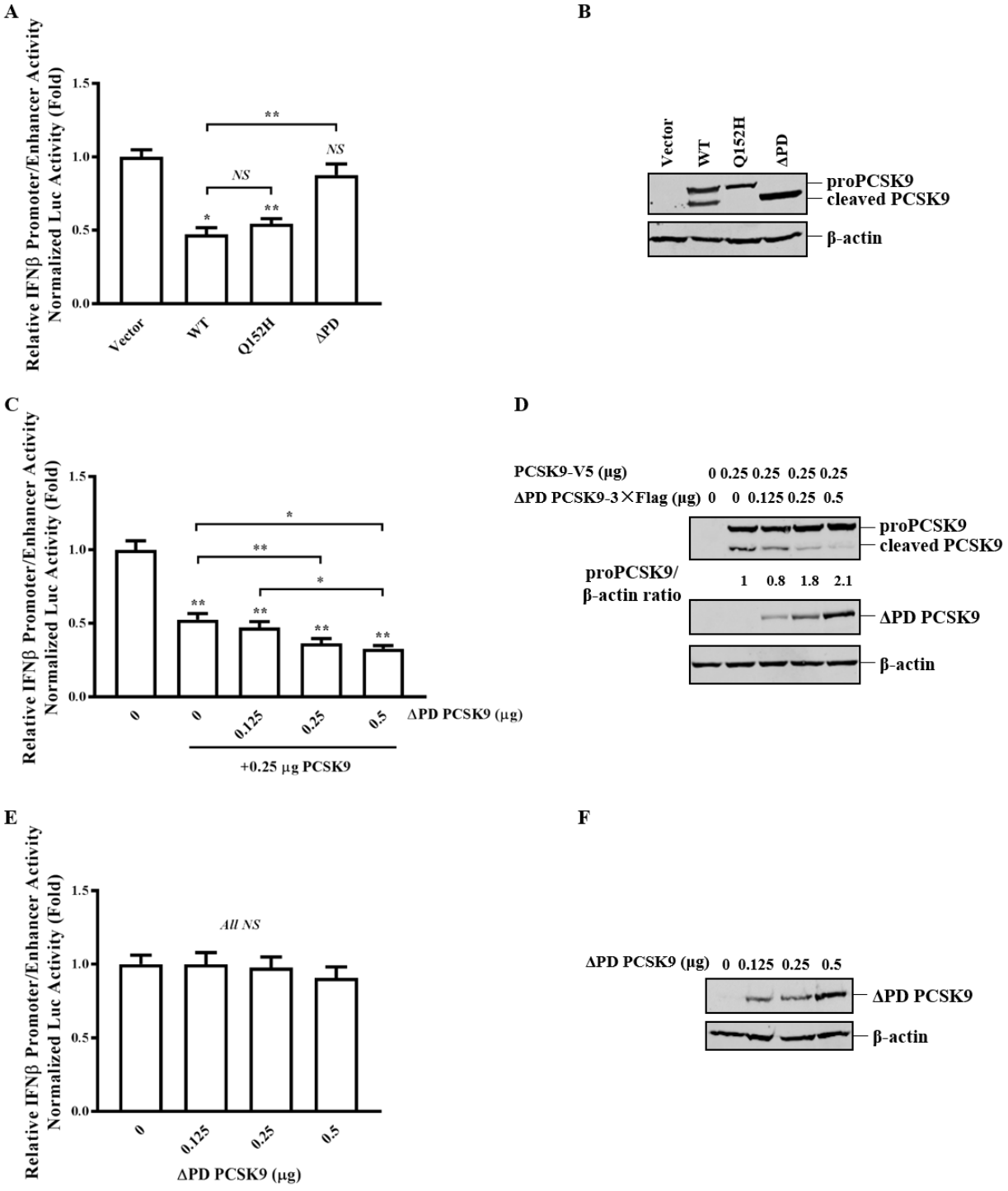
A
Relative IFNβ Promoter/Enhancer Activity
Normalized Luc Activity (Fold)
1.5
1.0
0.5
0.0
**
NS
NS
*
**
Vector
WT
Q152H
ΔPD
B
Vector
WT
Q152H
ΔPD
proPCSK9
cleaved PCSK9
β-actin
C
Relative IFNβ Promoter/Enhancer Activity
Normalized Luc Activity (Fold)
1.5
1.0
0.5
0.0
*
**
*
**
**
**
**
0
0
0.125
0.25
0.5
ΔPD PCSK9 (μg)
+0.25 μg PCSK9
D
PCSK9-V5 (μg) 0 0.25 0.25 0.25 0.25
ΔPD PCSK9-3×Flag (μg) 0 0 0.125 0.25 0.5
proPCSK9
cleaved PCSK9
proPCSK9/
β-actin ratio
1 0.8 1.8 2.1
ΔPD PCSK9
β-actin
E
Relative IFNβ Promoter/Enhancer Activity
Normalized Luc Activity (Fold)
1.5
1.0
0.5
0.0
All NS
0
0.125
0.25
0.5
ΔPD PCSK9 (μg)
F
ΔPD PCSK9 (μg) 0 0.125 0.25 0.5
ΔPD PCSK9
β-actin

Figure 5.2 ProPCSK9 down-regulates IFNβ promoter/enhancer activity. A, Huh-7 cells were co-transfected with -125/+19 IFNβ promoter/enhancer-luciferase reporter and vector, or plasmids expressing Flag-tagged wild-type, Q152H or ΔPD PCSK9. After 48 h, luciferase assay was performed. **B**, Cell lysates from (A) were subjected to Western blotting using Flag-tag and β-actin antibodies. **C**, Huh-7 cells were co-transfected with -125/+19 IFNβ promoter/enhancer-luciferase reporter, plasmids expressing V5-tagged PCSK9 and increasing amounts of Flag-tagged ΔPD PCSK9. After 48 h, luciferase assay was performed. **D**, Cell lysates from (C) were subjected to Western blotting using V5-tag, Flag-tag and β-actin antibodies. The band intensity of proPCSK9 was normalized to that of β-actin. **E**, Huh-7 cells were co-transfected with -125/+19 IFNβ promoter/enhancer-luciferase reporter and plasmids expressing increasing amounts of Flag-tagged ΔPD PCSK9. After 48 h, luciferase assay was performed. **F**, Cell lysates from (E) were subjected to Western blotting using Flag-tag and β-actin antibodies. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant.

5.6.3 PCSK9 suppresses IFNβ promoter/enhancer activation by the ATF-2/c-Jun complex

We showed that PCSK9 inhibited IFNβ promoter/enhancer activity via PRDIV (Figure 5.1E). This led us to speculate that ATF-2/c-Jun played a role in PCSK9-induced IFNβ promoter/enhancer down-regulation. ATF-2 and c-Jun are from the basic region-leucine zipper (bZIP) family that functions as dimers. They can form both homodimers and heterodimer with the heterodimer having a higher binding affinity to PRDIV than homodimers (Carrillo et al., 2010; Panne et al., 2004). We first confirmed the effect of ATF-2 and c-Jun on IFNβ transcription. Huh-7 cells were co-transfected with -125/+19 IFNβ promoter/enhancer-luciferase reporter and vector, plasmids expressing ATF-2, c-Jun, or ATF-2 and c-Jun. Luciferase assay result showed that either ATF-2 or c-Jun could up-regulate IFNβ promoter/enhancer activity (Figure 5.3A). Co-expression of ATF-2 and c-Jun further up-regulated IFNβ promoter/enhancer activity compared to ATF-2 or c-Jun alone. The expression of ATF-2 and c-Jun was demonstrated by Western blotting (Figure 5.3B). We then examined the effect of PCSK9 on IFNβ promoter/enhancer activity in the presence of ATF-2 and c-Jun. Our results showed that PCSK9 significantly decreased IFNβ promoter/enhancer activity than vector or EGFP controls (Figure 5.3C), suggesting that PCSK9 suppressed IFNβ promoter/enhancer activation by the ATF-2/c-Jun complex.

5.6.4 PCSK9 inhibits ATF-2/c-Jun dimerization and ATF-2/c-Jun binding to IFNβ enhancer

Next, we intended to investigate how PCSK9 inhibited IFNβ promoter/enhancer activation by the ATF-2/c-Jun complex. We hypothesized that protein-protein interaction might be a mechanism and therefore examined the interaction between PCSK9 and ATF-2, PCSK9 and c-Jun. Co-IP experiments showed that ATF-2 could interact with c-Jun or PCSK9, whereas c-Jun could not interact with PCSK9 (Figure 5.4A and B). As a negative control, EGFP could not interact with ATF-2 or c-Jun.

Since ATF-2 interacts with PCSK9, we were interested to determine whether this interaction affected ATF-2/c-Jun heterodimer formation. To test this hypothesis, we performed co-IP experiments by co-expressing Myc-tagged ATF-2, Flag-tagged c-Jun and V5-tagged PCSK9. When using Flag-tag antibody to immunoprecipitate c-Jun, less ATF-2 was detected in the

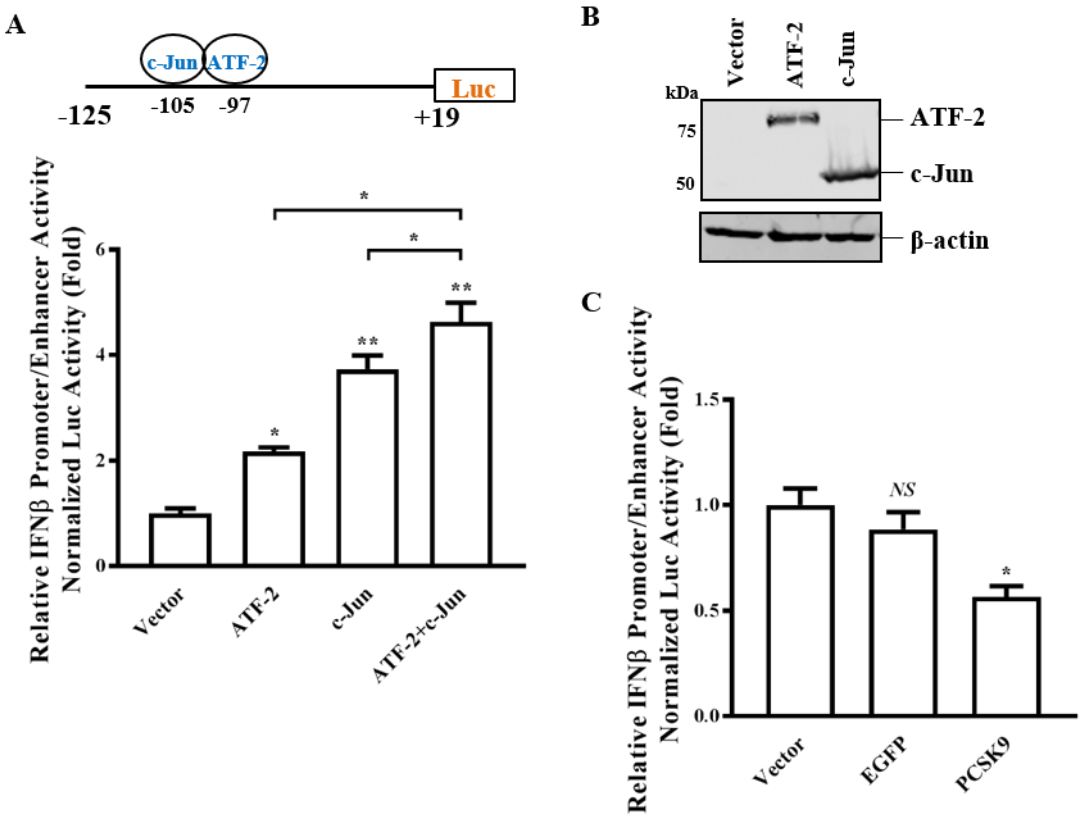

Figure 5.3 PCSK9 suppresses IFNβ promoter/enhancer activation by the ATF-2/c-Jun complex. A, The schematic of -125/+19 IFNβ promoter/enhancer-luciferase reporter with ATF-2/c-Jun binding site indicated. Huh-7 cells were co-transfected with -125/+19 IFNβ promoter/enhancer-luciferase reporter and vector, or plasmids expressing Myc-tagged ATF-2, c-Jun, or ATF-2 and c-Jun. After 48 h, luciferase assay was performed. **B**, Huh-7 cells were transfected with vector, or plasmids expressing Myc-tagged ATF-2, c-Jun. After 48 h, the cell lysates were subjected to Western blotting using Myc-tag and β-actin antibodies. **C**, Huh-7 cells were co-transfected with -125/+19 IFNβ promoter/enhancer-luciferase reporter, plasmids expressing Myc-tagged ATF-2, c-Jun and vector, Flag-tagged EGFP or PCSK9. After 48 h, luciferase assay was performed. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant.

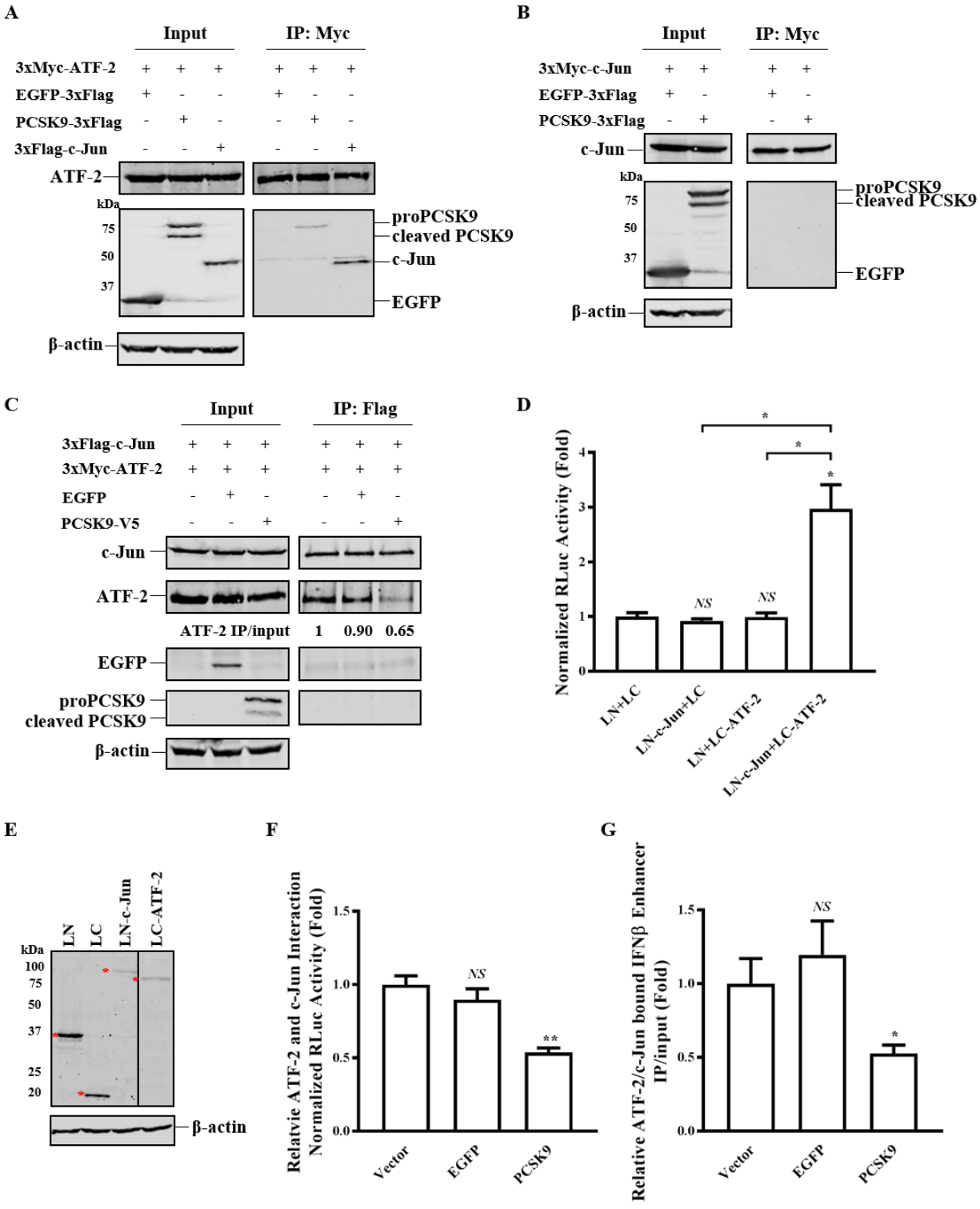
A
Input
IP: Myc
3xMyc-ATF-2
EGFP-3xFlag
PCSK9-3xFlag
3xFlag-c-Jun
ATF-2
kDa
75
50
37
proPCSK9
cleaved PCSK9
c-Jun
EGFP
β-actin
B
Input
IP: Myc
3xMyc-c-Jun
EGFP-3xFlag
PCSK9-3xFlag
c-Jun
kDa
75
50
37
proPCSK9
cleaved PCSK9
EGFP
β-actin
C
Input
IP: Flag
3xFlag-c-Jun
3xMyc-ATF-2
EGFP
PCSK9-V5
c-Jun
ATF-2
ATF-2 IP/input
1
0.90
0.65
EGFP
proPCSK9
cleaved PCSK9
β-actin
D
Normalized RLuc Activity (Fold)
NS
NS
LN+LC
LN-c-Jun+LC
LN+LC-ATF-2
LN-c-Jun+LC-ATF-2
E
LN
LC
LN-c-Jun
LC-ATF-2
kDa
100
75
50
37
25
20
β-actin
F
Relatvie ATF-2 and c-Jun Interaction
Normalized RLuc Activity (Fold)
NS
Vector
EGFP
PCSK9
G
Relative ATF-2/c-Jun bound IFNβ Enhancer
IP/input (Fold)
NS
Vector
EGFP
PCSK9

Figure 5.4 PCSK9 inhibits ATF-2/c-Jun dimerization and ATF-2/c-Jun binding to IFNβ enhancer. A and **B**, HEK293T cells were co-transfected with plasmids expressing Myc-tagged ATF-2 and Flag-tagged EGFP, PCSK9 or c-Jun (**A**), or Myc-tagged c-Jun and Flag-tagged EGFP or PCSK9 (**B**). After 48 h, the co-IP experiments were performed with Myc-tag antibody and then the eluted samples were subjected to Western blotting with Myc-tag, Flag-tag and β-actin antibodies. **C**, HEK293T cells were co-transfected with plasmids expressing Myc-tagged ATF-2, Flag-tagged c-Jun and EGFP or V5-tagged PCSK9. After 48 h, the co-IP experiment was performed with Flag-tag antibody and then the eluted samples were subjected to Western blotting with Flag-tag, Myc-tag, AFP/EGFP, V5-tag and β-actin antibodies. The band intensity of immunoprecipitated ATF-2 was normalized to that of input ATF-2. **D**, Huh-7 cells were co-transfected with plasmids expressing LN and LC, LN-Myc-c-Jun and LC, LN and LC-Flag-ATF-2, or LN-Myc-c-Jun and LC-Flag-ATF-2. After 48 h, luciferase assay was performed. **E**, Huh-7 cells were transfected with plasmids expressing LN, LC, LN-Myc-c-Jun or LC-Flag-ATF-2. After 48 h, the cell lysates were subjected to Western blotting using Myc-tag, Flag-tag and β-actin antibodies. Protein bands of interest were indicated by *. **F**, Huh-7 cells were co-transfected with plasmids expressing LN-Myc-c-Jun, LC-Flag-ATF-2 and vector, Flag-tagged EGFP or PCSK9. After 48 h, luciferase assay was performed. **G**, Huh-7 cells were co-transfected with -205/+19 IFNβ promoter/enhancer-luciferase reporter and plasmids expressing Myc-tagged ATF-2, Flag-tagged c-Jun and EGFP or V5-tagged PCSK9. After 48 h, the ChIP experiment was performed with Flag-tag antibody. The immunoprecipitated IFNβ promoter/enhancer was plotted versus input IFNβ promoter/enhancer. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant.

immunoprecipitates in PCSK9-tranfected cells in comparison to vector- and EGFP-transfected cells (Figure 5.4C), indicating that PCSK9 inhibited ATF-2 and c-Jun dimerization.

To substantiate the co-IP results, we also carried out SLCA to quantify ATF-2/c-Jun complex formation. Huh-7 cells were co-transfected with plasmids expressing N-terminus of Renilla luciferase fused with c-Jun (LN-c-Jun) and C-terminus of Renilla luciferase fused with ATF-2 (LC-ATF-2). The luciferase activity of LN-c-Jun/LC-ATF-2 pair significantly increased compared to control pairs LN/LC, LN-c-Jun/LC and LN/LC-ATF-2 (Figure 5.4D), suggesting an interaction between ATF-2 and c-Jun. The expression of LN, LC, LN-c-Jun and LC-ATF-2 was shown in Figure 5.4E. To test the effect of PCSK9 on the interaction between ATF-2 and c-Jun, Huh-7 cells were co-transfected with LN-c-Jun, LC-ATF-2 and vector, EGFP or PCSK9. PCSK9 significantly down-regulated luciferase activity compared to vector or EGFP (Figure 5.4F), indicating once again that PCSK9 inhibited ATF-2 and c-Jun dimerization.

The ATF-2/c-Jun heterodimer activates IFNβ transcription through binding to PRDIV in the IFNβ enhancer region (Ford and Thanos, 2010). We have demonstrated that PCSK9 interfered with ATF-2/c-Jun dimerization. We therefore reasoned that PCSK9 interacting with ATF-2 affected binding of the ATF-2/c-Jun complex to the IFNβ enhancer. A ChIP experiment was carried out to determine the amount of IFNβ enhancer that bound to ATF-2/c-Jun complex in the presence of PCSK9. Cells co-transfected with vector or EGFP were used as negative controls. Figure 5.4G showed that PCSK9 resulted in less IFNβ promoter/enhancer in the immunoprecipitates compared to the controls, indicating that PCSK9 suppressed ATF-2/c-Jun complex binding to the IFNβ enhancer.

Taken together, our results showed that the interaction between PCSK9 and ATF-2 inhibited ATF-2/c-Jun dimerization and ATF-2/c-Jun binding to IFNβ enhancer.

5.7 Discussion

In this study, we showed that PCSK9 decreases IFNβ expression as a novel function of PCSK9. We first demonstrated that ectopic expression of PCSK9 significantly reduces the mRNA and protein levels of IFNβ. We also demonstrated decreased level of the 2',5'-OAS-1 transcript, an ISG downstream of IFNβ signaling. Using IFNβ promoter/enhancer-luciferase reporters of different lengths, we mapped the PRDIV sequence as the region involved in IFNβ promoter/enhancer inhibition by PCSK9 in a dose-dependent manner.

PCSK9 presents as proPCSK9 and cleaved PCSK9 as a result of auto-cleavage. These two species may have different functions. For example, PCSK9 maturation and secretion is dependent on the auto-cleavage, and secreted PCSK9 functions in LDLR degradation (Poirier and Mayer, 2013). We demonstrated that proPCSK9, but not cleaved PCSK9, is able to inhibit IFNβ promoter/enhancer activity. These findings may indicate another functional segregation between intracellular and extracellular PCSK9.

We elucidated the mechanism of how PCSK9 down-regulates IFNβ promoter/enhancer activity involving the PRDIV region with a binding site for the ATF-2/c-Jun heterodimer. We found an interaction between PCSK9 and ATF-2 that interferes with ATF-2 and c-Jun dimerization, and consequently reduces ATF-2/c-Jun binding to the IFNβ enhancer.

Would these findings suggest any potential mechanisms of how PCSK9 inhibits HCV replication as we initially set out to investigate? It has been documented that both ATF-2 and c-Jun can regulate the transcription of a large number of genes involved in numerous cellular processes, such as cell cycle progression, cell proliferation and apoptosis (Lopez-Bergami et al., 2010). For example, ATF-2 has been shown to regulate the transcription of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) by binding to its promoter under hypoxia (Qian et al., 2012). Moreover, c-Jun has also been shown to regulate PTEN transcription (Hettinger et al., 2007). Interestingly, we recently demonstrated an inhibitory effect of PTEN on HCV replication (Wu et al., 2017). As such, it is possible that PCSK9 inhibits HCV replication by modulating the functions of ATF-2 and/or c-Jun in regulating PTEN level. This hypothesis should be tested in future studies.

Both PCSK9 and IFNβ are ubiquitously expressed. PCSK9 has been identified as an etiologic factor of atherosclerosis through at least two potential mechanisms (Wicinski et al., 2017). Aberrant functional PCSK9 levels result in increased lipid accumulation in circulation. PCSK9 also induces the expression of adhesion molecules as well as pro-inflammatory cytokines and chemokines (Ricci et al., 2018). These factors cause abnormal accumulation of immune cells in blood vessels, ultimately resulting in arterial lesions. The JAK/STAT signaling pathway, one of the major mechanisms for IFNβ to exert its functions, is involved in modulating inflammation (Qu et al., 2018). As such, whether PCSK9 contributes to atherosclerosis through regulating IFNβ expression awaits further investigation.

5.8 Conclusion

In conclusion, we demonstrated, for the first time, an inhibitory effect of PCSK9, a modulator of cholesterol homeostasis, on IFNβ expression. Given the approved clinical use of PCSK9 inhibitors in familial hypercholesterolemia patients, our findings should have important implications in optimizing this therapy.

5.9 Acknowledgements

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6.0 LINKER BETWEEN CHAPTERS 3.0, 5.0 AND 7.0

In Chapter 3.0, I demonstrated that PCSK9 inhibits HCV replication through the interaction with HCV NS5A. In Chapter 5.0, I showed that PCSK9 suppresses IFNβ expression, which indicates that PCSK9 may modulate HCV infection via regulating immune response. In these two chapters, I examined the effect of PCSK9 on HCV and revealed the mechanisms by which PCSK9 affects HCV. To elucidate the complex interactions between HCV and PCSK9, I studied the second objective that is to determine the effect of HCV on PCSK9 in Chapter 7.0.

7.0 HEPATITIS C VIRUS REGULATES PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9 PROMOTER ACTIVITY

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Keywords: HCV; PCSK9 promoter; SREBP; HNF-1; FoxO3; Sp1

Running title: **HCV regulates PCSK9 promoter**

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7.2 Authors' Contribution

All the experiments within this chapter were performed by Zhubing Li. The manuscript was written by Zhubing Li and edited by Qiang Liu.

7.3 Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secretory serine protease mainly expressed in the liver. Although PCSK9 has been shown to inhibit hepatitis C virus (HCV) entry and replication, whether HCV regulates PCSK9 transcription has not been well studied. PCSK9 promoter activity is modulated by numerous transcription factors including sterol-regulatory element binding protein (SREBP)-1a, SREBP-1c, SREBP-2, hepatocyte nuclear factor (HNF)-1, and forkhead box O3 (FoxO3). Since they are differently regulated by HCV, we studied the effects of these transcription factors on PCSK9 promoter activity in the context of HCV infection and replication. We demonstrated that PCSK9 promoter activity was up-regulated after HCV infection and in HCV genomic replicon cells. We also studied the effects of HCV proteins on the PCSK9 promoter activity. While HCV structural proteins core, E1 and E2 had no effect, nonstructural proteins NS2, NS3, NS3-4A, NS5A and NS5B enhanced, and p7 or NS4B decreased PCSK9 promoter activity. Furthermore, we showed that transcription factors SREBP-1c, HNF-1α and specificity protein 1 increased PCSK9 promoter activity in HCV replicon cells, whereas SREBP-1a, HNF-1β and FoxO3 had an inhibitory effect. These results demonstrated the molecular mechanisms of how HCV modulates PCSK9 promoter activity and advanced our understanding on the complex interactions between HCV and PCSK9.

7.4 Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9), a secretory serine protease in the proprotein convertase family, is mainly expressed in the liver, but also found in intestine, kidney and brain (Seidah et al., 2014; Seidah et al., 2003). Since it enhances lysosome-mediated degradation of low-density lipoprotein receptor (LDLR), it indirectly up-regulates low-density lipoprotein cholesterol (LDLC) in plasma and is associated with hypercholesterolemia (Melendez et al., 2017; Tibolla et al., 2011). PCSK9 is also involved in regulating lipoprotein synthesis, glucose metabolism, neurological function, inflammation and infection (Norata et al., 2016; Seidah et al., 2017). The level of PCSK9 is regulated by intracellular cholesterol concentration (Norata et al., 2014). Accordingly, numerous transcription factors involved in maintaining lipid homeostasis can regulate PCSK9 promoter activity. For example, sterol-regulatory element binding protein (SREBP)-1a, -1c, -2 and hepatocyte nuclear factor (HNF)-1α have been shown to differentially regulate PCSK9 transcription (Costet et al., 2006; Costet et al., 2008; Li et al., 2009). Other transcription factors, such as forkhead box O3 (FoxO3) and specificity protein 1 (Sp1), are also involved in regulating PCSK9 promoter activity (Chen et al., 2016; Jeong et al., 2008; Tao et al., 2013).

Hepatitis C virus (HCV) is a single-stranded RNA virus that affects about 2-3% world population and causes severe liver damage (Maasoumy and Wedemeyer, 2012; Tellinghuisen et al., 2007). It has seven genotypes and its positive-sense genome encodes a polyprotein. The polyprotein is processed to generate structural proteins core, envelope proteins E1 and E2, and nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Hundt et al., 2013; Moradpour and Penin, 2013; Smith et al., 2014). A complex interplay between HCV and host lipid metabolism has been identified during the HCV life cycle (Paul et al., 2014; Popescu et al., 2014). Accordingly, numerous viral proteins have been reported to be able to regulate transcription factors involved in maintaining host lipid homeostasis. HCV-3a core, 1a NS2, 1b, 2a and 3a NS4B, and 3a NS5A are reported to activate SREBP-1 (Jackel-Cram et al., 2010; Oem et al., 2008b; Park et al., 2009; Waris et al., 2007; Xiang et al., 2010). HCV-2a infection can induce SREBP-1, -2 and FoxO3 (Bose et al., 2014; Tikhanovich et al., 2014; Waris et al., 2007). Interestingly, different HCV genotypes show different regulatory effects on HNF-1. Qadri et al. demonstrated an up-regulation of HNF-1 by HCV-1b (Qadri et al., 2006), whereas Matsui et al.

revealed a down-regulation of HNF-1α by HCV-2a (Matsui et al., 2012). Taken together, these studies demonstrate that HCV utilizes multiple means to regulate transcription factors involved in host lipid metabolism.

We and others have demonstrated that PCSK9 can inhibit HCV entry and replication (Labonte et al., 2009; Li and Liu, 2018; Syed et al., 2014). However, the effect of HCV on PCSK9, especially on its promoter activity, has not been well characterized. As such, we studied the effect of HCV-2a on the PCSK9 promoter activity.

7.5 Material and Methods

7.5.1 Plasmids and reagent

Human PCSK9 -440 promoter (-440 to -94)-luciferase reporter, wild-type or with sterol-regulatory element (SRE) or HNF-1 binding sequence mutations were received from Dr. Liu (Li et al., 2009). PCSK9 -440 promoter-luciferase reporters containing Sp1 binding site mutations were received from Dr. Park (Jeong et al., 2008). To generate plasmids expressing HCV-2a proteins or glutathione S-transferase (GST) with a Flag tag, the respective coding sequences were amplified by PCR using plasmid pFLneo-J6/JFH-1(p7-RLuc-2A) provided by Dr. Rice (Jones et al., 2007) or pGEX-5X-1 (GE Healthcare Life Sciences) as the templates and cloned into the pEF vector. Plasmids expressing Flag-tagged SREBP-1a, SREBP-1c, SREBP-2 and dominant negative (DN) SREBP-1 were described previously (Shi et al., 2016). Plasmids expressing GST, GST-DN Sp1, or full-length Sp1 were received from Drs. Thiel and Suske, respectively (Petersohn and Thiel, 1996; Sapetschnig et al., 2004; Thiel and Cibelli, 1999). Plasmids expressing Myc-tagged Sp1 was generated by cloning the Sp1 cDNA with a Myc-tag into pcDNA3.1 vector (Thermo Fisher Scientific). FR_HNF-1α (Senkel et al., 2005) and FR_HNF-1β (Thomas et al., 2004) were gifts from Dr. Ryffel (Addgene plasmids #31104 and #31101). The coding sequences of HNF-1α and HNF-1β were cloned into the p3xFlag CMV7.1 vector (Sigma-Aldrich), resulting in plasmids expressing Flag-tagged HNF-1α and HNF-1β. A DN HNF-1α-expressing plasmid was generated by cloning the coding sequence of aa.1-280 (Watanabe et al., 2007) into the p3xFlag CMV7.1 vector. Flag-FoxO3 was a gift from Dr. Greenberg (Addgene plasmid #8360) (Brunet et al., 2004). Sequences encoding full-length or aa. 1-304 (DN) (Olagnier et al., 2014; Reed et al., 2012; van Grevenynghe et al., 2008) FoxO3 were also cloned into the p3xFlag CMV7.1 vector.

7.5.2 Cell lines, transfection, HCV infection

Huh-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich) at 37°C and 5% CO_2. Huh-7 replicon cells with HCV-2a J6/JFH-1(p7-RLuc2A) or HCV-2a J6 core^{Flag}/JFH-1(p7-RLuc-2A) replicating RNAs were described previously (Hundt et al., 2015; Wu et al., 2017). DNA transfection was carried out using calcium phosphate or the jetPEI reagent (Polyplus) according to the manufacturer's protocol. Huh-7 cells were infected with cell-culture derived HCV-2a J6 core^{Flag}/JFH-1(p7-RLuc-2A) (HCVcc) as previously described (Li and Liu, 2018). Mithramycin A (Sigma-Aldrich) was used as previously described (Xiang et al., 2010).

7.5.3 Luciferase assay and Western blotting

Cells were lysed in Passive Lysis Buffer (Promega) or SDS sample buffer 48 h after transfection. Luciferase assay and Western blotting were performed as previously described (Li and Liu, 2018). The antibodies used were: GST, Myc, β-actin (Cell Signaling Technology), Flag (Sigma-Aldrich), and secondary antibodies IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

7.5.4 Statistical analysis

All experiments were performed in triplicate. The luciferase assay data were analyzed using GraphPad Prism 7. Statistical differences were determined by Student's *t*-test or one-way analysis of variance (ANOVA), and indicated as * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant.

7.6 Results and Discussion

7.6.1 HCV up-regulates PCSK9 promoter activity

To determine the effect of HCV infection on PCSK9 promoter activity, Huh-7 cells were transfected with PCSK9 -440 promoter-luciferase reporter and then infected with HCVcc at 8 h after transfection. Luciferase assay performed at 48 h after infection demonstrated significantly higher luciferase activity conferred by the PCSK9 -440 promoter after HCV infection in comparison to mock infection (Figure 7.1A). This result indicated that HCV infection up-

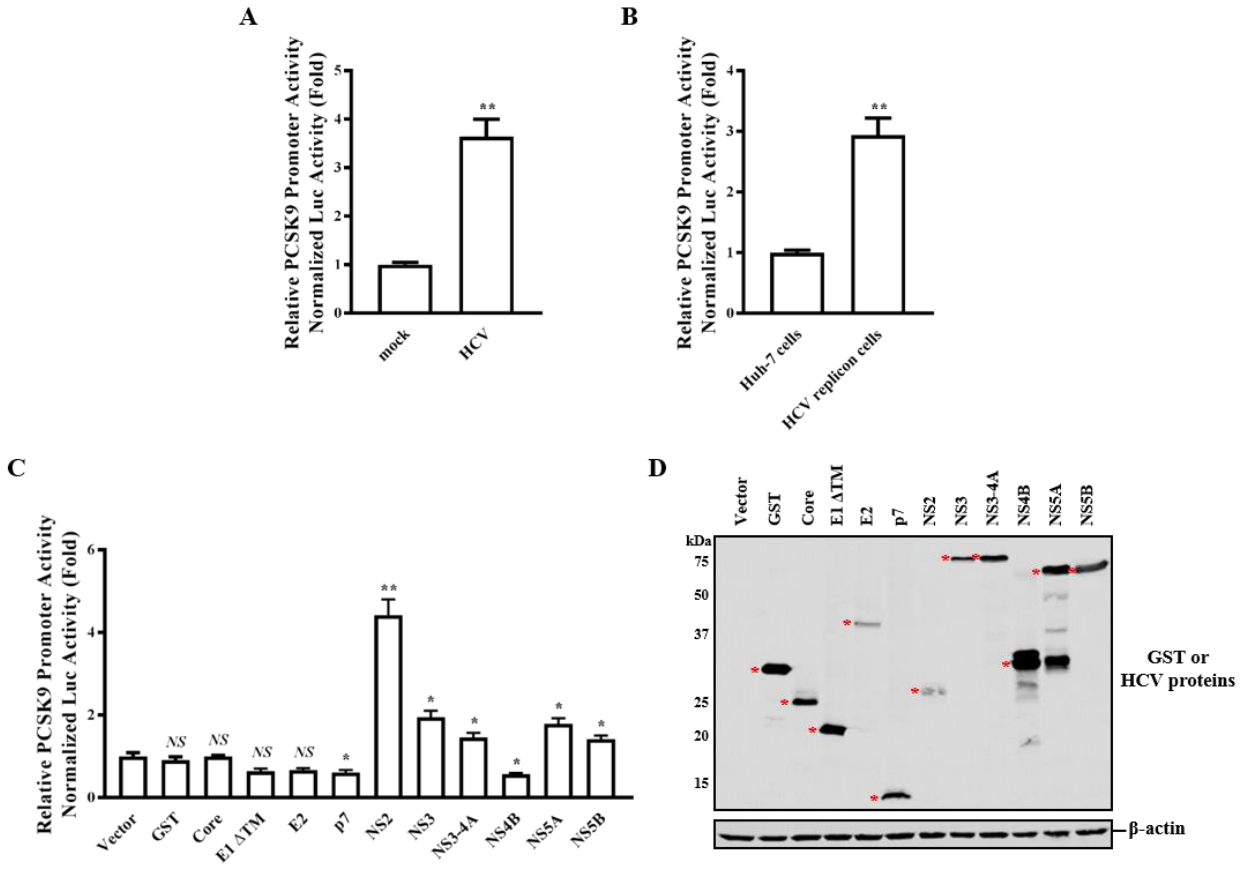

Figure 7.1 HCV up-regulates PCSK9 promoter activity. A, Huh-7 cells were transfected with PCSK9 -440 promoter-luciferase reporter for 8 h and infected with HCVcc at an MOI of 0.05. **B**, Huh-7 cells and HCV-2a replicon cells were transfected with PCSK9 -440 promoter-luciferase reporter. **C**, Huh-7 cells were co-transfected with PCSK9 -440 promoter-luciferase reporter and vector, or plasmids expressing Flag-tagged GST or Flag-tagged HCV proteins. Luciferase assay was performed 48 h after infection (A) or transfection (B and C). Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant. **D**, Cell lysates from (C) were subjected to Western blotting with Flag and β-actin antibodies. The bands of GST and HCV proteins were indicated by *.

regulates PCSK9 promoter activity.

To determine whether HCV replication is sufficient to activate PCSK9 promoter, we transfected Huh-7-HCV-2a J6/JFH-1(p7-RLuc2A) genomic replicon cells with the PCSK9 -440 promoter-luciferase reporter plasmid. Huh-7 cells were also transfected as a control. Figure 7.1B showed that PCSK9 -440 promoter activity was elevated in HCV replicon cells compared to that in Huh-7 cells, indicating PCSK9 promoter activation by HCV replication.

Then we studied the effects of HCV proteins on the PCSK9 promoter activity. For this purpose, we cloned the coding sequences for individual HCV proteins in an expression vector. While the expression of all the other HCV proteins could be readily demonstrated by Western blotting after transfection, the expression of the E1 protein could only be detected after deleting the C-terminal transmembrane domain (ΔTM, Δaa. 159-192) (Falson et al., 2015) (Figure 7.1D and data not shown). To determine the effects of HCV proteins on PCSK9 promoter activity, Huh-7 cells were co-transfected with plasmids expressing HCV proteins and the PCSK9 -440 promoter-luciferase reporter. Plasmid vector and GST-expressing plasmid were used as controls. Figure 7.1C showed that PCSK9 promoter activity increased when co-transfected with NS2, NS3, NS3-4A, NS5A or NS5B, while p7 or NS4B expression inhibited PCSK9 promoter activity. The three structural proteins had no effect. These results indicated that HCV proteins that differently regulated PCSK9 promoter activity. Future studies are required to elucidate the underlying mechanisms by investigating the regulatory effects of HCV proteins on the transcription factors involved in PCSK9 transcription.

In summary, HCV infection and replication could up-regulate PCSK9 promoter activity with the involvement of several HCV proteins.

7.6.2 The role of SREBPs in PCSK9 promoter regulation by HCV

The PCSK9 -440 promoter contains SRE (-345 to -337) sequence and SREBPs have been shown to regulate PCSK9 promoter activity (Jeong et al., 2008). Given the fact that HCV can up-regulate the activity of SREBPs, we hypothesized that SREBPs were involved in PCSK9 promoter activation by HCV. To test this, we used a mutant PCSK9 -440 promoter without the SRE sequence for transfection into HCV replicon cells. As shown in Figure 7.2A, luciferase activity conferred by the PCSK9 -440 mSRE promoter decreased significantly than the wild-type, suggesting that the SRE sequence is essential.

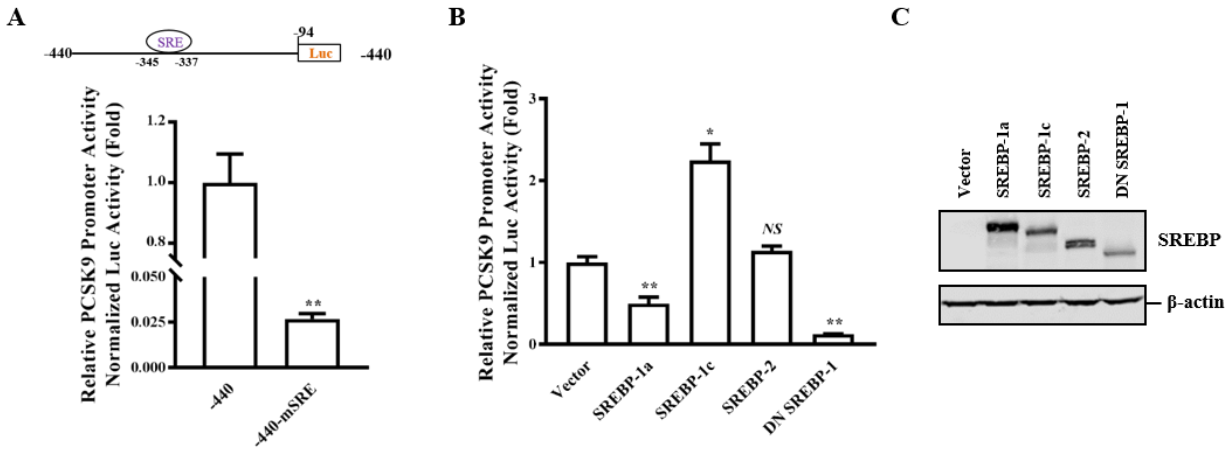

Figure 7.2 SREBP-1a and -1c have opposite effects on the PCSK9 promoter activity in HCV replicon cells. A, HCV-2a replicon cells were transfected with wild-type or SRE sequence mutated PCSK9 -440 promoter-luciferase reporters. SRE sequence within PCSK9 -440 promoter is indicated schematically. **B**, HCV-2a replicon cells were co-transfected with PCSK9 -440 promoter-luciferase reporter and vector or plasmids expressing Flag-tagged SREBPs. Luciferase assay was performed 48 h after transfection. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant. **C**, Cell lysates from (B) were subjected to Western blotting with Flag and β-actin antibodies.

There are three SREBP proteins: SREBP-1a, SREBP-1c and SREBP-2 (Shimano and Sato, 2017). SREBP-1a and -1c only differ in the N-terminal transactivation domain, whereas SREBP-2 is a separate protein (Shimano and Sato, 2017; Xiao and Song, 2013). To determine the effect of individual SREBPs on PCSK9 promoter activation by HCV, we co-transfected HCV replicon cells with plasmids expressing SREBPs with the PCSK9 -440 promoter-luciferase reporter and measured luciferase activities. As shown in Figure 7.2B, SREBP-1c up-regulated PCSK9 promoter activity compared to vector control, whereas SREBP-1a down-regulated PCSK9 promoter activity and SREBP-2 had no effect. DN SREBP-1 that lacks the N-terminal transactivation domain further decreased the PCSK9 promoter activity (Figure 7.2B). The expression of SREBP proteins was demonstrated by Western blotting (Figure 7.2C). These results indicated that SREBP-1c is involved on PCSK9 promoter activation by HCV.

7.6.3 The effects of HNF-1α, -1β and FoxO3 on PCSK9 promoter regulation by HCV

In addition to the SRE sequence, the PCSK9 -440 promoter has the binding sequence for HNF-1 (-386 GTTAATGTTTAAT -374) (Chen et al., 2016; Li et al., 2009). A FoxO3 binding motif, -381 TGTTTA -376, was also identified within the HNF-1 binding sequence (Chen et al., 2016). As such, we were interested in determining the involvements of HNF-1 and FoxO3 in PCSK9 promoter activation by HCV.

To study the effect of HNF-1, we transfected a mutant PCSK9 -440 promoter with three nucleotide substitutions (underlined) in the HNF-1 binding sequence (-386 TGGAATGTTTAAT -374) (Li et al., 2009) into HCV replicon cells and measured luciferase activity. As shown in Figure 7.3A, PCSK9 -440 mHNF-1 promoter gave rise to significantly lower luciferase activity than wild-type, indicating that the HNF-1 binding sequence is required. HNF-1 has two isoforms: HNF-1α and HNF-1β (Costa et al., 2003). To confirm the finding obtained from promoter mutant (Figure 7.3A) and also determine the effects of the two HNF-1 isoforms, we measured PCSK9 promoter activity after ectopic expression of HNF-1α and HNF-1β in HCV replicon cells. Our results showed that HNF-1α enhanced, whereas HNF-1β down-regulated PCSK9 promoter activity (Figure 7.3B). DN HNF-1 with the C-terminal transactivation domain deletion inhibited PCSK9 promoter activity even further than HNF-1β (Figure 7.3B). The expression of HNF-1s was demonstrated by Western blotting (Figure 7.3C). These results indicated that HNF-1α, but not HNF-1β, is involved in PCSK9 promoter activation by HCV.

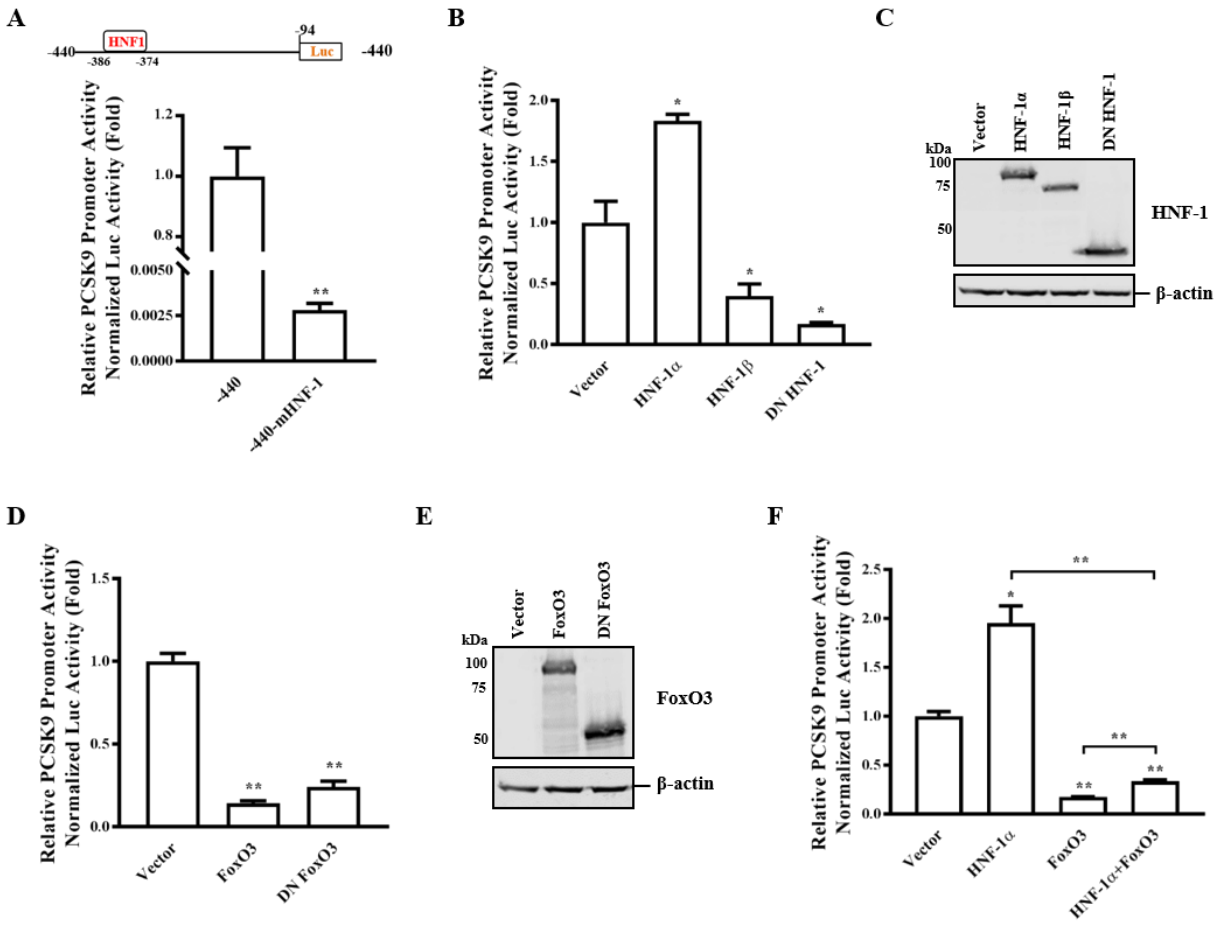

Figure 7.3 HNF-1α elevates PCSK9 promoter activity, while HNF-1β and FoxO3 suppress PCSK9 promoter activity in HCV replicon cells. A, HCV-2a replicon cells were transfected with wild-type or HNF-1 binding site mutated PCSK9 -440 promoter-luciferase reporters. HNF-1 binding site within PCSK9 -440 promoter is indicated schematically. **B**, **D** and **F**, HCV-2a replicon cells were co-transfected with PCSK9 -440 promoter-luciferase reporter and vector or plasmids expressing Flag-tagged HNF-1s (**B**), Flag-tagged FoxO3s (**D**), or HNF-1, FoxO3, HNF-1 and FoxO3 (**F**). Luciferase assay was performed 48 h after transfection. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$ or ** if $p < 0.01$. **C** and **E**, Cell lysates from (B and D) were subjected to Western blotting with Flag and β-actin antibodies.

FoxO3 can epigenetically suppress PCSK9 transcription by recruiting histone deacetylase sirtuin 6 (Tao et al., 2013). To study the role of FoxO3 in PCSK9 promoter activation by HCV, HCV replicon cells were co-transfected with plasmid expressing FoxO3 and the PCSK9 -440 promoter-luciferase reporter. Luciferase assay results showed that FoxO3 significantly inhibited PCSK9 promoter activity (Figure 7.3D). DN FoxO3 with C-terminal transactivation domain truncation also showed a decrease in PCSK9 promoter activity, but the reduction level was less compared to wild-type FoxO3 (Figure 7.3D). The expression of FoxO3 and DN FoxO3 were demonstrated by Western blotting (Figure 7.3E). These results indicated that FoxO3 down-regulates PCSK9 promoter activity in HCV replicon cells.

The binding sequences for HNF-1 and FoxO3 overlap within the PCSK9 promoter. Our results so far showed opposite effects of HNF-1α and FoxO3 on PCSK9 promoter activity in HCV replicon cells. As such, we were interested in determining whether a competition and/or dominance exists between these two factors. For this purpose, HCV replicon cells were co-transfected with plasmids expressing HNF-1α and FoxO3, together with the PCSK9 -440 promoter-luciferase reporter. Our results showed an intermediate phenotype upon co-expression of HNF-1α and FoxO3 in comparison to ectopic expression of either factor alone (Figure 7.3F). FoxO3 drastically ablated PCSK9 promoter activation by HNF-1α to a level that was significantly lower than vector control. On the other hand, HNF-1α partially alleviated the suppression of PCSK9 promoter activity by FoxO3. Our results suggested a dominant role of FoxO3 when both HNF-1α and FoxO3 were present and provided additional support to the hypothesis that FoxO3 can prevent HNF-1α binding to the PCSK9 promoter (Chen et al., 2016).

7.6.4 Sp1 is involved in PCSK9 promoter regulation by HCV

There are five putative Sp1 motifs in the proximal PCSK9 -440 promoter (Figure 7.4A) (Jeong et al., 2008). To identify the role of these Sp1 motifs in PCSK9 promoter activity by HCV, we transfected HCV replicon cells with PCSK9 promoter-luciferase reporters that contain different Sp1 motif mutations. Luciferase assay showed that Sp1 motif 1, 2 and 5 mutants significantly down-regulated PCSK9 promoter activity, whereas Sp1 motifs 3 and 4 mutants had no effects (Figure 7.4A). These results indicated that three out of five Sp1 binding motifs are involved in PCSK9 promoter activation in HCV replicon cells.

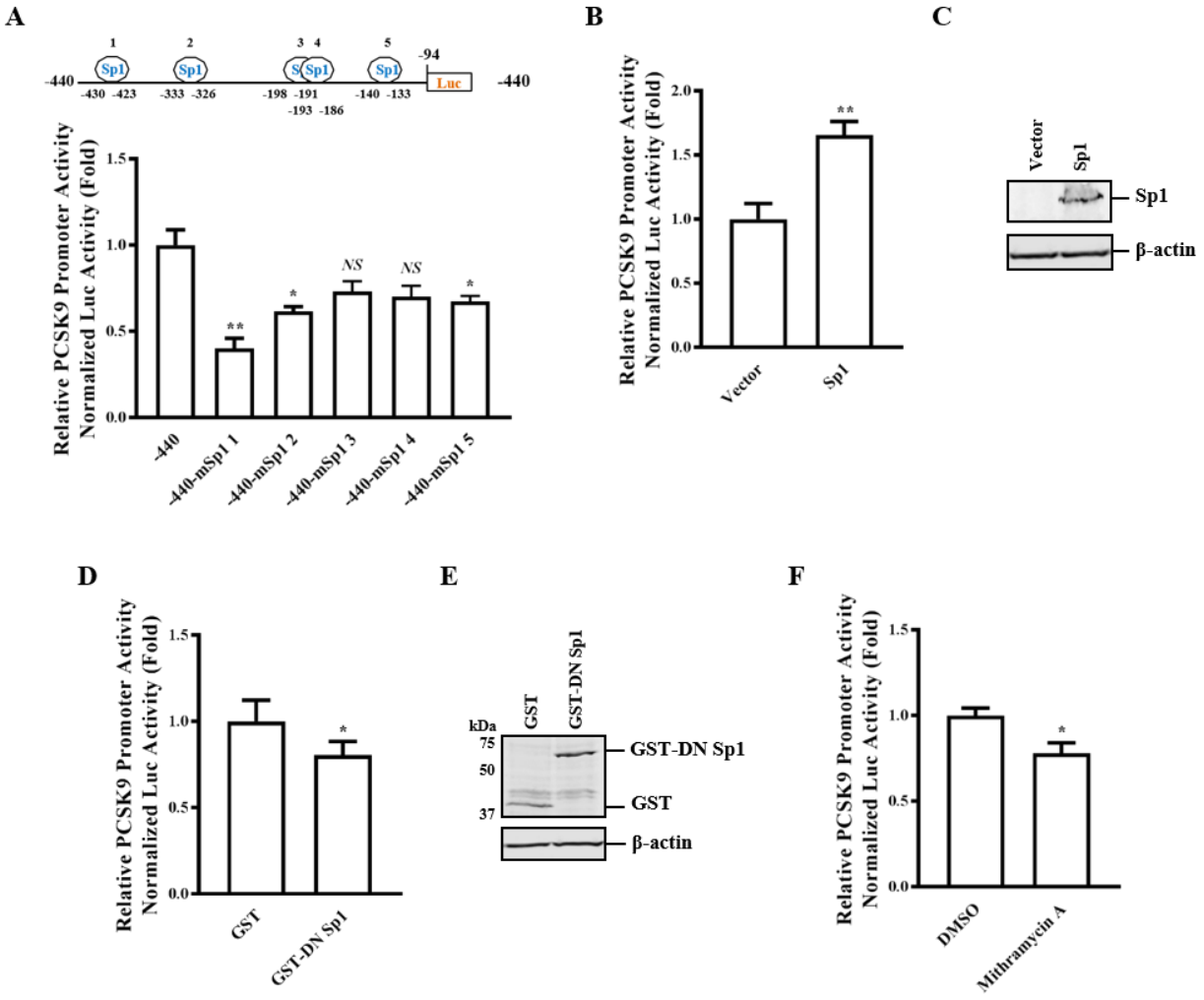

Figure 7.4 Sp1 up-regulates PCSK9 promoter activity in HCV replicon cells. A, HCV-2a replicon cells were transfected with wild-type or different Sp1 binding sites mutated PCSK9 -440 promoter-luciferase reporters. Five Sp1 binding sites within PCSK9 -440 promoter are indicated schematically. **B** and **D**, HCV-2a replicon cells were co-transfected with PCSK9 -440 promoter-luciferase reporter and vector or plasmids expressing Myc-tagged Sp1 (**B**), GST or GST-tagged DN Sp1 (**D**). Luciferase assay was performed 48 h after transfection. **C** and **E**, Cell lysates from (B and D) were subjected to Western blotting with Myc, GST and β-actin antibodies. **F**, HCV-2a replicon cells were transfected with PCSK9 -440 promoter-luciferase reporter for at 36 h and treated with 100 nM mithramycin A or DMSO for 12 h before luciferase assay was performed. Statistical differences between samples were demonstrated as follows: * if $p < 0.05$, ** if $p < 0.01$, or *NS* for not significant.

To further demonstrate the role of Sp1 in PCSK9 promoter activation by HCV, we measured PCSK9 promoter activity after ectopic expression of wild-type or DN Sp1 in HCV replicon cells. Our results showed that wild-type Sp1 could up-regulate PCSK9 -440 promoter compared to vector control (Figure 7.4B). DN Sp1 could modestly, but significantly, decrease PCSK9 -440 promoter activity (Figure 7.4D). The expression of Sp1 proteins was demonstrated by Western blotting (Figure 7.4C and E). Furthermore, inhibition of Sp1 activity by mithramycin A also significantly decreased PCSK9 -440 promoter activity in HCV replicon cells (Figure 7.4F). These results collectively indicated that Sp1 is involved in PCSK9 promoter activation by HCV.

In conclusion, we demonstrated that HCV infection and replication could enhance PCSK9 promoter activity. Furthermore, HCV proteins either increased or decreased, or did not affect the PCSK9 promoter activity. We also demonstrated differential effects of several transcription factors on PCSK9 promoter regulation in HCV replicon cells. SREBP-1c, HNF-1α and Sp1 enhanced, while SREBP-1a, HNF-1β and FoxO3 suppressed PCSK9 promoter activity. SREBP-2 had no effect. Our results suggested a rather complex mechanism underlying the regulation of PCSK9 promoter activity by HCV.

PCSK9 has been shown to be able to inhibit HCV entry and replication (Labonte et al., 2009; Li and Liu, 2018; Syed et al., 2014). Combining with our data, it is conceivable that there are complex interactions between PCSK9 and HCV. HCV can up-regulate PCSK9 expression, which in turn inhibits HCV propagation. However, HCV infection can induce LDLR expression (Syed et al., 2014) and LDLR is important for both HCV entry and replication (Albecka et al., 2012). Given the fact that the major role of PCSK9 is LDLR down-regulation, we envision a complex interplay among HCV, LDLR and PCSK9.

7.7 Acknowledgements

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8.0 GENERAL DISCUSSION AND CONCLUSION

8.1 General Discussion

Previous research about PCSK9 mainly focuses on its function in regulating lipid homeostasis. In this thesis, I focused on the role of PCSK9 in antiviral activity. I first studied the effect of PCSK9 on the HCV life cycle and elucidated the possible mechanism by which PCSK9 down-regulates HCV replication. To further investigate the antiviral mechanism, I examined whether PCSK9 affected innate immunity through regulating IFN expression. In addition, HCV regulates several transcription factors that are involved in PCSK9 expression, which led me to study how HCV modulated PCSK9 expression. The complex interactions between HCV and PCSK9 were revealed in my study (Figure 8.1).

In Chapter 3.0, I examined the effect of PCSK9 on different stages of the HCV life cycle expect for HCV entry. The role of PCSK9 in HCV entry has been studied previously. PCSK9 can prevent HCV entry through down-regulating HCV receptors LDLR and CD81 (Labonte et al., 2009). Since the HCV life cycle is closely related with host lipid metabolism (Popescu et al., 2014; Schaefer and Chung, 2013) and PCSK9 regulates lipid homeostasis (Horton et al., 2007), I hypothesized that PCSK9 regulates different stages of the HCV life cycle. In this chapter, I showed that PCSK9 does not have effect on HCV translation, virion assembly or secretion (Figure 3.1 and 3.S1), but it inhibits viral replication (Figure 3.2, 3.S2, 3.3 and 3.S3). Although PCSK9 down-regulates HCV replication, it is not due to PCSK9-induced LDLR degradation (Figure 3.2E 3.S2C). It is not consistent with previous finding that gain-of-function mutant PCSK9 induces slightly more inhibitory effect on HCV replication (Syed et al., 2014). My results suggest that LDLR degradation mediated by PCSK9 is not involved in HCV replication and the lipid regulation function of PCSK9 is not involved in HCV translation, virion assembly or secretion.

When I tried to identify the mechanism by which PCSK9 inhibits HCV replication, I hypothesized that it might result from the interaction between PCSK9 and HCV proteins. Since NS5A is essential for HCV replication and can regulate HCV replication through interacting with many cellular proteins (Cordek et al., 2011; Ross-Thriepland and Harris, 2015), I carried out the further study on the interaction between PCSK9 and NS5A. I found that PCSK9 directly interacts with NS5A through NS5A domain I aa. 95-215 (Figure 3.6), and showed the role of this region in

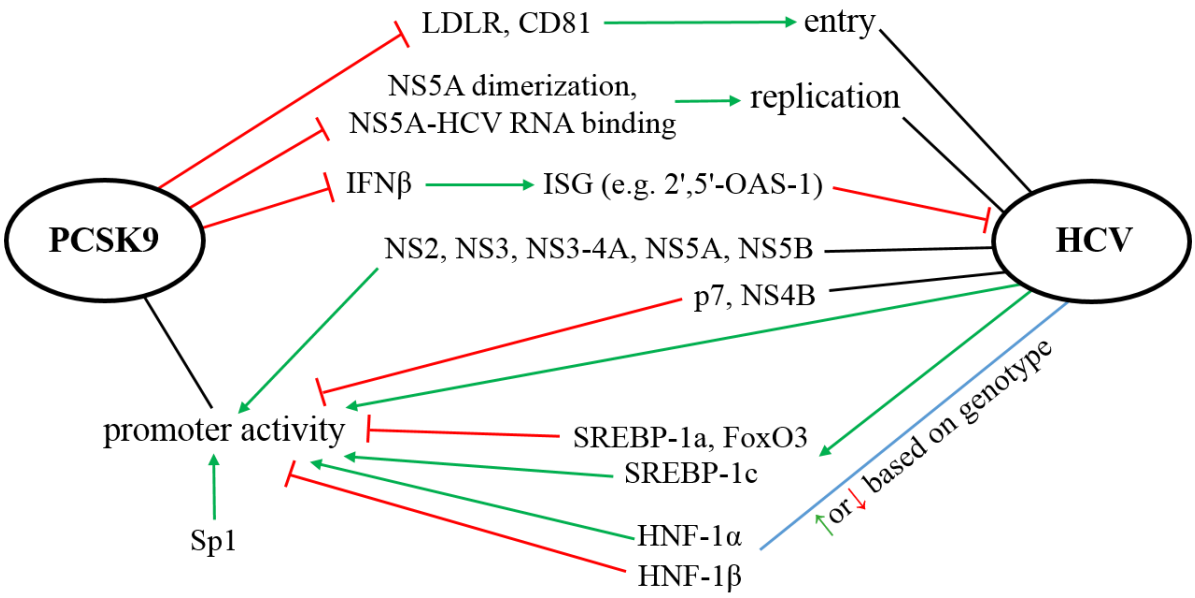

Figure 8.1 The complex interactions between HCV and PCSK9. PCSK9 inhibits HCV entry by down-regulating LDLR and CD81, and inhibits HCV replication by preventing NS5A dimerization and NS5A-HCV RNA binding. PCSK9 down-regulates IFNβ expression through interacting with ATF-2, which may facilitate HCV infection. HCV up-regulates PCSK9 promoter activity. HCV viral proteins and several transcription factors that can be regulated by HCV differently modulate PCSK9 promoter activity.

NS5A dimerization, NS5A-RNA binding and HCV replication (Figure 3.7). Then I demonstrated that the interaction with PCSK9 diminishes NS5A dimerization and NS5A-HCV RNA binding (Figure 3.8). Since NS5A dimerization and NS5A-HCV RNA binding are critical for HCV replication (Lim et al., 2012), I concluded that PCSK9 inhibits HCV replication through the interaction with NS5A.

Figure 3.8C showed that PCSK9 inhibits NS5A and HCV RNA binding. Except for the blockage of HCV RNA binding region in NS5A by PCSK9, it possibly results from HCV RNA binding to PCSK9. I used RPISeq web server (http://pridb.gdcb.iastate.edu/RPISeq) (Muppirala et al., 2011) to predict HCV RNA and PCSK9 interaction by inputting HCV RNA sequence and PCSK9 protein sequence. The predicted interaction probability is greater than 0.5, which is considered as positive. It suggests that PCSK9 is likely to interact with HCV RNA. I also performed the electrophoretic mobility shift assay (EMSA) using HCV 3'UTR RNA and purified PCSK9 protein. The preliminary data showed that there might be interaction between HCV 3'UTR RNA and PCSK9 (data not shown). Therefore, PCSK9 inhibits HCV replication may be due to PCSK9 binding to HCV RNA.

In Chapter 3.0, I showed that PCSK9 has an inhibitory effect on HCV replication. Since IFN is important to provide an antiviral state upon HCV infection (Heim, 2013) and the link between PCSK9 and IFN has not been studied, I was curious to know whether PCSK9 could induce IFN production to inhibit HCV infection. However, instead of an up-regulation, I showed a down-regulation of IFNβ promoter/enhancer activity, mRNA and protein levels by PCSK9 overexpression (Figure 5.1). It suggested that PCSK9 can potentially facilitate HCV infection, which is opposite from what I expected. In this thesis, I demonstrated that PCSK9 has contradictory effects on HCV infection. It can not only reduce HCV replication through interacting with HCV NS5A, but also promote HCV infection by down-regulating IFNβ expression. It is not clear how PCSK9 balances these two different effects during HCV infection. But overall it seems that the inhibitory effect plays a dominant role since PCSK9 induces a reduction in HCV replication in HCV replicon cells and HCVcc-infected cells (Figure 3.2 and 3.S2).

It has been reported that NS5A down-regulates type I IFN signaling by inhibiting STAT1 phosphorylation through protein-protein interaction (Kumthip et al., 2012; Lan et al., 2007). NS5A can not only interfere with several ISGs through protein-protein interaction including

affecting protein kinase R (PKR) activation and 2',5'-OAS function, but also suppress expression of ISGs by up-regulating IL-8 (Gale et al., 1998; Polyak et al., 2001; Taguchi et al., 2004). Besides, NS5A can interact with TLR adaptor myeloid differentiation primary response 88 (MyD88), which prevents the activation of TLR signaling pathway and its downstream cytokine production (Abe et al., 2007). All these suggest an up-regulation of HCV replication by NS5A through suppressing innate immunity. I have showed that NS5A suppresses IFNβ promoter/enhancer activity (data not shown), which also indicates that NS5A counteracts IFN signaling pathway induced by HCV infection. However, the mechanism by which NS5A regulates IFNβ promoter/enhancer activity is not known. NS5A can up-regulate NF-κB expression and promote its translocation to the nucleus via increasing nitric oxide synthase 2A (NOS2A) (Jiang et al., 2011). It can also activate NF-κB through phosphorylating its inhibitory subunit nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) at Tyr^{42} and Tyr^{305} and promoting IκBα degradation (Waris et al., 2003). Park et al. showed that NS5A can reduce NF-κB activation stimulated by tumor necrosis factor (TNF)-α and TNF receptor-associated factor 2 (TRAF2) via interacting with TRAF2 (Park et al., 2002), whereas NS5A can activate c-Jun N-terminal kinase (JNK) mediated by TNF-α and TRAF2 via interacting with TRAF2 (Park et al., 2003). JNK can phosphorylate ATF-2 and c-Jun, thus activating their transcription activities (Hayakawa et al., 2004). Another study showed that NS5A decreases the transcription activity of activating protein-1 (AP-1) via Ras-extracellular signal-regulated kinase (ERK) pathway (Macdonald et al., 2003). AP-1 is a homodimeric or heterodimeric transcription factor and its subunits are from the bZIP family that contains ATF-2 and c-Jun (Shaulian and Karin, 2002). Since NF-κB, ATF-2 and c-Jun are the transcription factors that can bind to IFNβ enhancer to activate IFNβ expression (Ford and Thanos, 2010), NS5A might modulate IFNβ expression through regulating these transcription factors. When co-expressing PCSK9 and NS5A, I observed a further reduction of IFNβ promoter/enhancer activity compared to expressing PCSK9 or NS5A alone (data not shown). It implies that PCSK9 and NS5A have additive effect on inhibiting IFNβ promoter/enhancer. The interaction between PCSK9 and NS5A may also play a role in this process.

I also examined the interaction between PCSK9 and other HCV proteins except for NS5A, and found that PCSK9 can interact with several HCV proteins, including core, E1, E2, NS3, NS3-4A and NS5B (data not shown). HCV core is an RNA-binding protein that constitutes the

viral nucleocapsid and participates in infectious virion assembly. Arg^{70} and/or Leu^{91} mutation results in IFN therapy resistance among genotype 1b patients (Dubuisson, 2007; Khaliq et al., 2011b). E1 and E2 are envelope glycoproteins involved in HCV entry, virion assembly, secretion and immune evasion through glycosylation (Vieyres et al., 2014; Zeisel et al., 2011). NS3-4A contains serine protease activity and helicase activity, and plays an important role in HCV replication, virion assembly and immune evasion through the cleavage of MAVS and TRIF (Frick, 2006; Morikawa et al., 2011). NS5B is an RdRp essential for viral replication (Ranjith-Kumar and Kao, 2006) and its RdRp activity is essential for IFNβ induction (Moriyama et al., 2007). Since core, E1, E2, NS3-4A and NS5B all correlate with immune response, the interaction between PCSK9 and these viral proteins may further modulate immune response upon viral infection, thereby affecting HCV replication. PCSK9 also interacts with NS3-4A and NS5B that play an important role in HCV replication, suggesting it may regulate the function of NS3-4A and NS5B in HCV replication through protein-protein interaction. These could be other possible mechanisms by which PCSK9 inhibits HCV replication. Besides, E1 and E2 take part in HCV entry. Their interactions with PCSK9 may partially explain previous finding that PCSK9 inhibits HCV entry. In Figure 3.1B and C, I showed that PCSK9 does not affect virion assembly or secretion. However, PCSK9 can interact with core, E1, E2, NS3-4A and NS5A that are involved in virion assembly and/or secretion. It indicates that the interaction with PCSK9 has no effect on the functions of these viral proteins in virion assembly and/or secretion.

In Chapter 3.0 and 5.0, I showed that only proPCSK9 functions in regulating HCV replication (Figure 3.4 and 3.S4) and IFNβ promoter/enhancer activity (Figure 5.2). PCSK9 auto-cleavage produces the cleaved mature PCSK9, and then mature PCSK9 is secreted and targets LDLR for lysosomal degradation (Poirier and Mayer, 2013). It suggests that proPCSK9 and cleaved PCSK9 have distinct functions and auto-cleavage may regulate different functions of PCSK9. I also revealed that cleaved PCSK9 negatively regulates the auto-cleavage of proPCSK9 (Figure 3.4D and 5.2D). It implies the coexistence of proPCSK9 and cleaved PCSK9 in cells. There is a balance between proPCSK9 and cleaved PCSK9.

In Chapter 3.0, I demonstrated that PCSK9 inhibits HCV replication through the interaction with HCV NS5A (Figure 3.8), and PCSK9 and NS5A co-localize in the cytoplasm (Figure 3.5E). In Chapter 5.0, I showed that PCSK9 suppresses IFNβ promoter/enhancer activity via interacting with ATF-2 (Figure 5.4). HCV replication takes place in membranous web that is derived from

and partially associated with the ER membrane (Meyers et al., 2016). DNA transcription takes place in the nucleus (Thomas and Chiang, 2006). ATF-2 contains the nuclear localization signal (NLS) and nuclear export signal (NES), and is present in both nucleus and cytoplasm (Watson et al., 2017). Except for the ER, proPCSK9 localization has not been examined. I predicted the NLS and NES within PCSK9 using PCSK9 protein sequence by cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and LocNES predication tool (http://prodata.swmed.edu/LocNES/LocNES.php), respectively (Kosugi et al., 2009; Xu et al., 2015). Classic NLS is composed of monopartite or bipartite short sequences of basic amino acids. Monopartite NLS is characterized as a consensus sequence of Lys-Lys/Arg-X-Lys/Arg, while bipartite NLS contains two short sequences of basic amino acids that are linked by a short spacer sequence (Bauer et al., 2015; Lange et al., 2007). NES is a leucine-rich sequence defined as Leu-X_{2-3}-Leu-X_{2-3}-Leu-X-Leu, where leucine can be replaced by isoleucine, valine, phenylalanine or methionine (Kutay and Guttinger, 2005). In Figure 8.2, there is one predicted NLS (indicated in red) and six predicted NESs (indicated in blue) in full-length PCSK9. The predicted NLS presents in CHRD. The first four predicted NESs are overlapping and located within the first 30 amino acids of the SP that are cleaved by signal peptidase after translation, thus do not present in proPCSK9. The last two predicted overlapping NESs are in PD. Therefore, there is one predicted NLS and two predicted NESs in proPCSK9, implying that proPCSK9 may shuttle between nucleus and cytoplasm like ATF-2. It makes me to speculate whether the interaction between PCSK9 and ATF-2 happens in the nucleus or in the cytoplasm and the role of different located proPCSK9 may be different.

In Chapter 7.0, I demonstrated that PCSK9 promoter activity is up-regulated through HCV infection and in HCV replicon cells (Figure 7.1A and B). To investigate the mechanism, I first examined the role of HCV viral proteins in PCSK9 promoter activity. NS2, NS3, NS3-4A, NS5A and NS5B up-regulate, and p7 and NS4B down-regulate PCSK9 promoter activity (Figure 7.1C). Then I found transcription factors SREBP-1c, HNF-1α and Sp1 increase, and SREBP-1a, HNF-1β and FoxO3 decrease PCSK9 promoter activity (Figure 7.2, 7.3 and 7.4). Since SREBPs, HNF-1 and FoxO3 can be regulated by HCV (Bose et al., 2014; Matsui et al., 2012; Qadri et al., 2006; Waris et al., 2007), it is more complicated that how HCV modulate PCSK9 expression (Figure 8.1).

1-	MGTVSSRRSW	WPLPLLLLLL	LLLGPAGARA	QEDEDGDYEE	LVLALRSEED	-50
51-	GLAEAPEHGT	TATFHRCAKD	PWRLPGTYVV	VLKEETHLSQ	SERTARRLQA	-100
101-	QAARRGYLTK	ILHVFHGLLP	GFLVKMSGDL	LELALKLPHV	DYIEEDSSVF	-150
151-	AQSIPWNLER	ITPPRYRADE	YQPPDGGSLV	EVYLLDTSIQ	SDHREIEGRV	-200
201-	MVTDFENVPE	EDGTRFHRQA	SKCDSHGTHL	AGVVSGRDAG	VAKGASMRSL	-250
251-	RVLNCQGKGT	VSGTLIGLEF	IRKSQLVQPV	GPLVVLLPLA	GGYSRVLNAA	-300
301-	CQRLARAGVV	LVTAAGNFRD	DACLYSPASA	PEVITVGATN	AQDQPVTLGT	-350
351-	LGTNFGRCVD	LFAPGEDIIG	ASSDCSTCFV	SQSGTSQAAA	HVAGIAAMML	-400
401-	SAEPELTLAE	LRQRLIHFSA	KDVINEAWFP	EDQRVLTPNL	VAALPPSTHG	-450
451-	AGWQLFCRTV	WSAHSGPTRM	ATAVARCAPD	EELLSCSSFS	RSGKRRGERM	-500
501-	EAQGGKLVCR	AHNAFGGEGV	YAIARCCLLP	QANCSVHTAP	PAEASMGTRV	-550
551-	HCHQQGHVLT	GCSSHWEVED	LGTHKPPVLR	PRGQPNQCVG	HREASIHASC	-600
601-	CHAPGLECKV	KEHGIPAPQE	QVTVACEEGW	TLTGCSALPG	TSHVLGAYAV	-650
651-	DNTCVVRSRD	VSTTGSTSEG	AVTAVAICCR	SRHLAQASQE	LQ	-692

Figure 8.2 PCSK9 protein sequence. NLS and NESs within PCSK9 are predicted using cNLS Mapper and LocNES predication tool, respectively. The predicted NLS is indicated in red and NESs are indicated in blue with underlines to specify overlapping sequences.

Although HCV induces PCSK9 promoter activity (Figure 7.1A and B), there is no significant difference in PCSK9 mRNA and protein levels between Huh-7 cells and HCV replicon cells (data not shown), suggesting that HCV does not significantly alter PCSK9 at mRNA and protein levels. It is not consistent with my PCSK9 promoter activity data. To elucidate the contradictory results, I tested the effect of HCV on PCSK9 3'UTR using different 3'UTR truncated constructs of PCSK9 promoter-luciferase-3'UTR reporters. The presence of two AUUUA AU-rich sequences in the extreme end of PCSK9 mRNA 3'UTR implies the instability of PCSK9 mRNA (Seidah et al., 2014). However, luciferase assay showed that PCSK9 3'UTR truncated constructs do not have significant effect on PCSK9 expression mediated by HCV (data not shown), which suggests that PCSK9 3'UTR is not involved in PCSK9 expression regulation by HCV.

Epigenetics is another possible mechanism of this inconsistence, which is defined as phenotype variations caused by external factors through regulating gene expression without changing DNA sequence. The well-known epigenetic mechanisms include DNA methylation, histone modifications and miRNA regulation (Goldberg et al., 2007; Handel et al., 2010). Histone acetylation regulation of PCSK9 promoter has been reported. Histone H4 acetylation on PCSK9 promoter mediated by HINFP, HINFP cofactor NPAT and HAT cofactor TRRAP can up-regulate PCSK9 expression (Li and Liu, 2012). Histone H3 deacetylation on PCSK9 promoter by FoxO3-recruited HDAC sirtuin6 can inhibit PCSK9 expression (Tao et al., 2013). Besides, it has been shown that HDAC activity increases in HCV replicon cells (Miura et al., 2008) and HCV core protein can interact with HAT p300/cAMP-response element-binding protein-binding protein (CBP) and enhance its HAT activity (Gomez-Gonzalo et al., 2004). Therefore, I hypothesized that the apparent discrepancy between PCSK9 promoter activity and mRNA level might result from differential epigenetic controls of the exogenously transfected and endogenous PCSK9 promoters. DNA methylation and histone acetylation inhibitors are widely used to study the role of epigenetics in gene expression regulation. I carried out RT-qPCR in Huh-7 cells and HCV replicon cells that were treated with different concentrations of 5-Aza-2'-deoxycytidine (Aza), HATIIi and nicotinamide, respectively. Aza is an inhibitor of DNA methyltransferase that induces hypomethylated DNA to re-express silenced hypermethylated genes (Poirier et al., 2014). HATIIi is a HAT inhibitor mainly targets to p300/CBP (Costi et al., 2007). Nicotinamide is an HDAC inhibitor that can inhibit sirtuins (Wang et al., 2012). Aza negatively regulates PCSK9

transcription at low concentrations, especially in HCV replicon cells (data not shown). HATIIi inhibits PCSK9 transcription in a dose-dependent manner, but induces more reduction of PCSK9 mRNA levels in Huh-7 cells (data not shown). Nicotinamide down-regulates PCSK9 transcription in HCV replicon cells at low concentrations, but does not have effect in Huh-7 cells (data not shown). These results show that DNA methylation or histone acetylation differently regulates PCSK9 transcription in Huh-7 cells and HCV replicon cells, and the relationship among epigenetics, HCV and PCSK9 expression should be determined in future studies.

In addition, it is reported that nuclear receptors LXR, PPARα and PPARγ are involved in PCSK9 expression (Costet et al., 2006; Duan et al., 2012). LXRα expression is elevated in HCV-1b core- or NS5A-expressing cells, HCV-1b replicon cells and chronic HCV infection patients (Garcia-Mediavilla et al., 2012; Lima-Cabello et al., 2011). The expression of PPARα and PPARγ is lower in chronic HCV genotype 3 infection compared to that in chronic HCV genotype 1 infection, and PPARγ can be suppressed by HCV-3a core (de Gottardi et al., 2006). So studying the effect of LXR, PPARα and PPARγ on PCSK9 expression in the context of HCV infection may further elucidate how HCV regulates PCSK9 expression.

However, Syed et al. showed there is no significant difference in PCSK9 mRNA level and a down-regulation of PCSK9 protein level in HCVcc-2a-infected Huh-7 cells compared to mock-infected cells (Syed et al., 2014). A clinical study reported plasma PCSK9 concentration in HCV genotype 1 and 3 chronic infection patients for the first time. In HCV genotype 3 patients, there are negative correlations between viral load and LDLC concentration, LVP load and ApoE, LVP ratio (LVP/(LVP+non-LVP)) and PCSK9 concentration. PCSK9 concentrations are significantly lower compared to HCV negative population (Bridge et al., 2015). In contrast, HCV genotype 1 patients present positive correlations between LVP load and ApoE, LVP ratio and PCSK9 concentration. Significantly higher PCSK9 concentrations are measured compared to HCV negative population (Bridge et al., 2015; Sheridan et al., 2012). Unlike in HCV negative population, PCSK9 concentration and total cholesterol or LDLC level are not correlated in HCV genotype 1 or 3 patients. Besides, there is no significant difference in PCSK9/LDLC ratio in these two HCV genotypes (Bridge et al., 2015). Therefore, HCV genotype 1 and 3 infections interfere with LDLC homeostasis and differently regulate plasma PCSK9 concentration. Taking together, the regulation of PCSK9 expression by HCV may be genotype-specific.

In Chapter 3.0, I demonstrated that PCSK9 down-regulates HCV replication. Considering that PCSK9 also plays an inhibitory role in HCV entry, PCSK9 may be an anti-HCV drug candidate. Since PCSK9 overexpression may result in hypercholesterolemia (Lambert et al., 2009) and the prevalence of liver steatosis among hypercholesterolemia patients is about 24% (Browning, 2006), the use of PCSK9 as antiviral to treat HCV patients may increase the risk of hypercholesterolemia and liver steatosis. PCSK9 may be selectively used to treat non-hypercholesterolemia and certain HCV genotype-infected patients.

8.2 Conclusion

In Chapter 2.0, I came up with the hypothesis that HCV can regulate PCSK9 expression, which in turn impacts different stages of the HCV life cycle. In the following chapters, I studied this hypothesis with two objectives, and demonstrated how HCV modulates PCSK9 promoter activity and how PCSK9 modulates HCV infection. The conclusions are as follows.

Objective 1: Study the role of PCSK9 in different stages of the HCV life cycle

(1) Identify the effect of PCSK9 on HCV translation, replication, assembly and secretion

- PCSK9 does not affect HCV translation, virion assembly or secretion.
- PCSK9 inhibits HCV replication in a dose-dependent manner.

(2) Identify the mechanism if PCSK9 affects certain stage of HCV life cycle

- PCSK9-induced LDLR degradation is not involved in down-regulating HCV replication.
- The uncleaved proPCSK9, but not cleaved PCSK9, inhibits HCV replication, suggesting that the auto-cleavage of PCSK9 affects HCV replication.
- PCSK9 interacts with HCV NS5A through NS5A domain I aa. 95-215, and this region is important for NS5A dimerization, NS5A-HCV RNA binding and HCV replication.
- The interaction between PCSK9 and NS5A suppresses NS5A dimerization and NS5A-HCV RNA binding, which could explain how PCSK9 inhibits HCV replication.
- PCSK9 inhibits IFNβ promoter/enhancer activity, mRNA and protein levels, as well as IFNβ signaling downstream ISG 2',5'-OAS-1 mRNA level. It indicates that PCSK9 may modulate HCV infection via regulating immune response.

- The uncleaved proPCSK9, but not cleaved PCSK9, inhibits IFNβ promoter/enhancer activity, suggesting that PCSK9 auto-cleavage plays a role in regulating IFNβ promoter/enhancer activity.
- PCSK9 down-regulates IFNβ promoter/enhancer activity via ATF/c-Jun complex.
- PCSK9 can interact with ATF-2, but cannot interact with c-Jun.
- PCSK9 suppresses ATF-2/c-Jun dimerization and ATF-2/c-Jun binding to IFNβ enhancer, indicating that the interaction between PCSK9 and ATF-2 contributes to PCSK9-induced IFNβ promoter/enhancer activity inhibition.

Objective 2: Determine the effect of HCV on PCSK9 expression

- PCSK9 promoter activity is up-regulated through HCV infection and in HCV replicon cells.
- HCV NS2, NS3, NS3-4A, NS5A and NS5B enhance, while p7 or NS4B decreases PCSK9 promoter activity.
- SREBP-1c, HNF-1α and Sp1 increase PCSK9 promoter activity in HCV replicon cells, whereas SREBP-1a, HNF-1β and FoxO3 have an inhibitory effect.
- FoxO3 plays a dominant role in modulating PCSK9 promoter activity when co-expressing HNF-1α and FoxO3 in HCV replicon cells.

8.3 Future Directions

The RNA-protein interaction prediction RPISeq web server and preliminary EMSA result suggested that HCV RNA and PCSK9 may interact. To identify whether the interaction between HCV RNA and PCSK9 leads to the suppression of HCV replication, I will further study PCSK9-HCV RNA binding, and examine the binding affinity between HCV RNA and NS5A, HCV RNA and PCSK9.

Several studies have shown that NS5A can regulate transcription factors NF-κB, ATF-2 and c-Jun that are involved in binding to IFNβ enhancer. I will focus on the mechanism by which NS5A down-regulates IFNβ expression. I will also investigate how the interaction between PCSK9 and NS5A affects IFNβ expression.

I have identified that PCSK9 can interact with HCV core, E1, E2, N3-4A and NS5B. Among these viral proteins, core, E1, E2, NS3-4A and NS5B play an immunoregulatory role,

while NS3-4A and NS5B are essential for HCV replication. Therefore, the functional significance of the interaction between PCSK9 and these HCV proteins needs to be further addressed. I will identify the specific interacting regions and examine how these interactions affect immune response and HCV replication.

In Chapter 3.0 and 5.0, I showed that PCSK9 inhibits HCV replication and IFNβ promoter/enhancer activity through protein-protein interaction and only proPCSK9 functions in these processes. Since HCV replication and IFNβ transcription happen in different compartments in cells and there is predicted NLS and NESs within proPCSK9, I am interested to detect the subcellular localization of proPCSK9. I will take the advantage of Q152H PCSK9 mutant and detect whether proPCSK9 presents in the nucleus. I will also co-express Q152H PCSK9 and ATF-2, and examine their co-localization.

In Chapter 5.0, I demonstrated there is interaction between PCSK9 and ATF-2. But I have not identified the specific region within ATF-2 that can interact with PCSK9. I will map the PCSK9-interacting region in ATF-2 and identify if this region overlaps with c-Jun-binding region or PRDIV-binding region, which will provide more evidence of how PCSK9 suppresses IFNβ promoter/enhancer activity.

I showed inconsistent results of exogenously transfected PCSK9 promoter activity, endogenous mRNA and protein levels in HCV replicon cells compared to Huh-7 cells. Besides, the preliminary epigenetics data are inconclusive. Nuclear receptors LXR, PPARα and PPARγ that regulate PCSK9 expression can be modulated by HCV. I will try to further identify the regulatory mechanism of endogenous PCSK9 expression in the context of HCV infection. Since HCV may regulate PCSK9 expression in a genotype-specific manner, I will also test the effect of different HCV genotypes on PCSK9 expression.

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